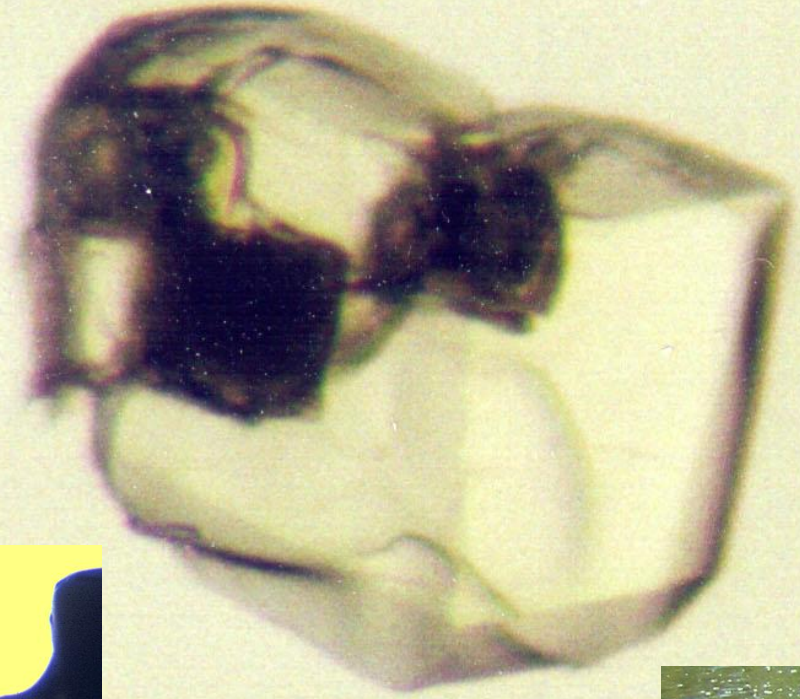
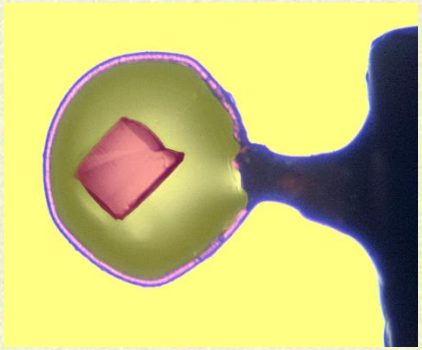
CRYO-COOLING and RADIATION DAMAGE



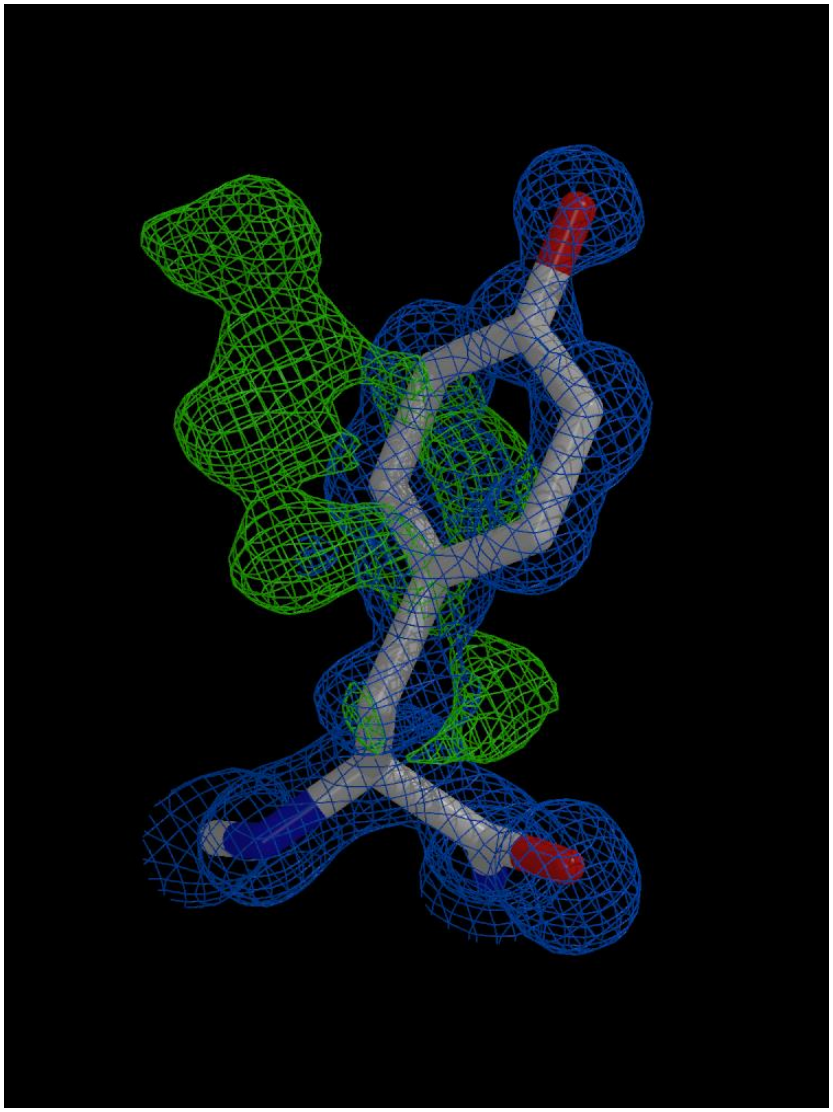
CCP4/DLS Workshop, December 2016



Elspeth Garman,
LMB, Oxford
Elspeth.garman@bioch.ox.ac.uk

Data quality pivotally affects the amount of biological information we obtain.

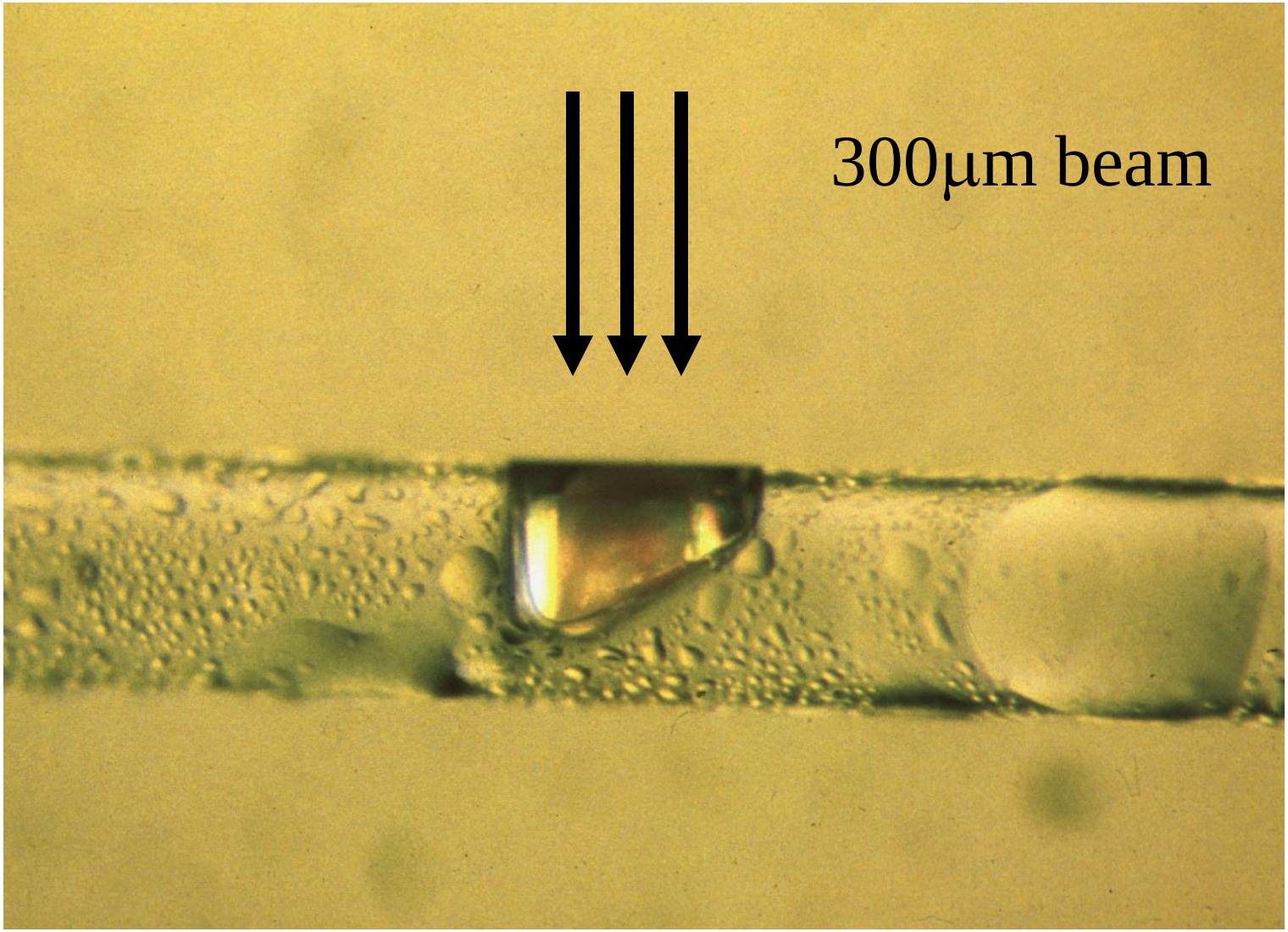
Farside
copyrighted cartoon



Detailed biological information

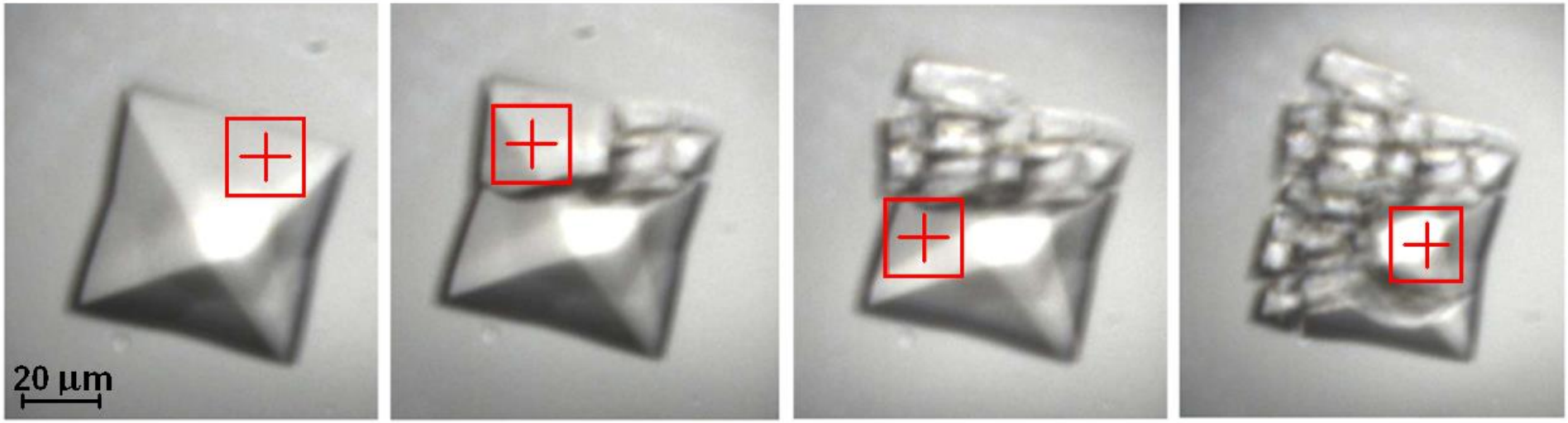
The Plan:

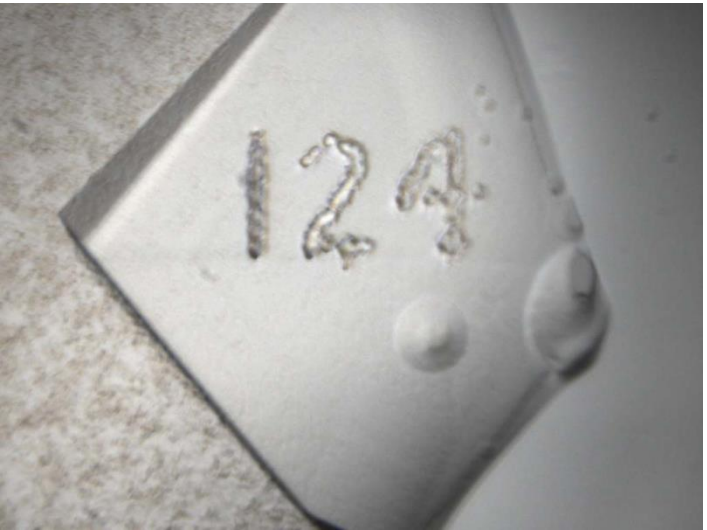
- Cryo techniques
  - **Why cool? Radiation damage.**
  - Optimising cryoprotection.
  - Testing at room temperature.
  - Storage and retrieval.
  - If nothing works…



Room temperature: HEWL crystal after 3 hours in a 2nd generation synchrotron beam.

I24, Diamond, *in situ* data collection from a Bovine Enterovirus 2 crystal, room temperature, 0.5 s 20 μm x 20 μm beam





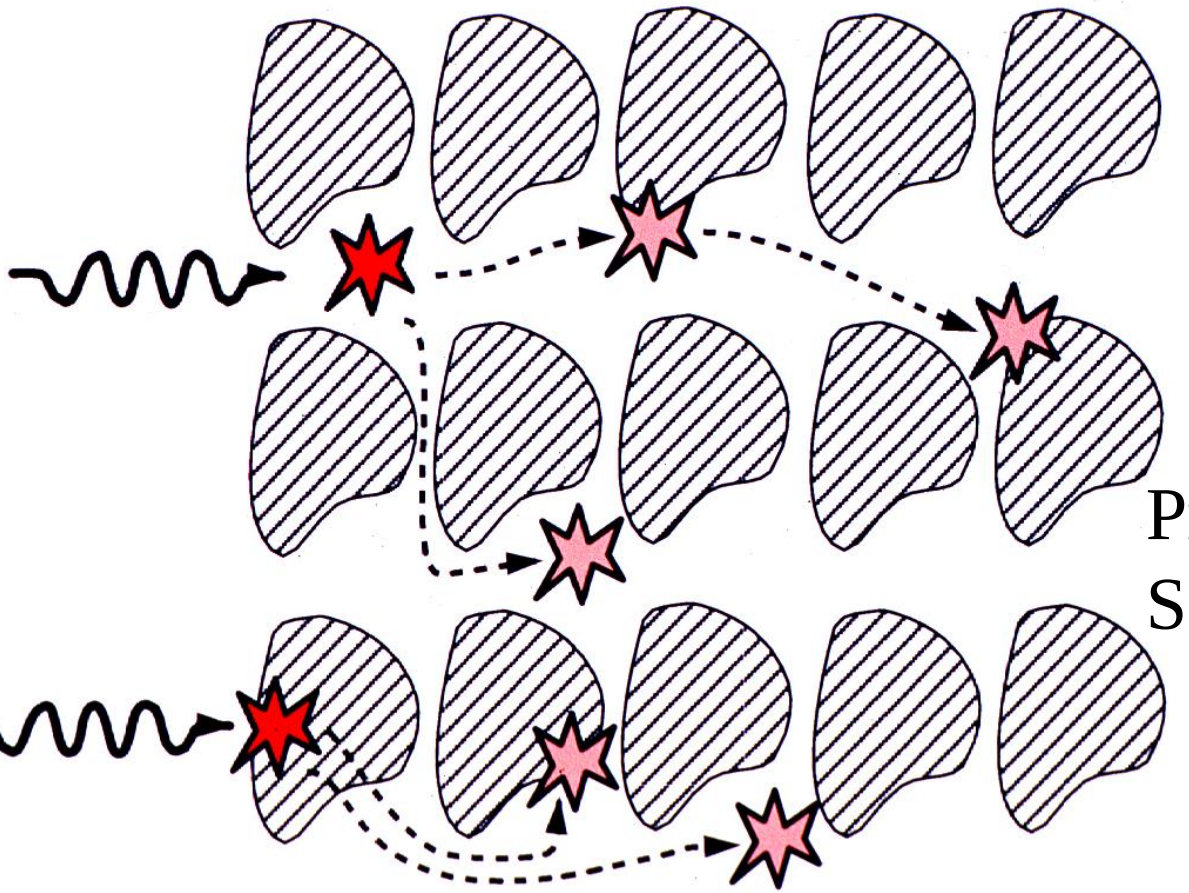
Axford *et al.*,
Acta Cryst D (2012) 592

Beamline logo I24
(Gwyndaf Evans *et al.*)

Radiation Damage

Primary

Secondary

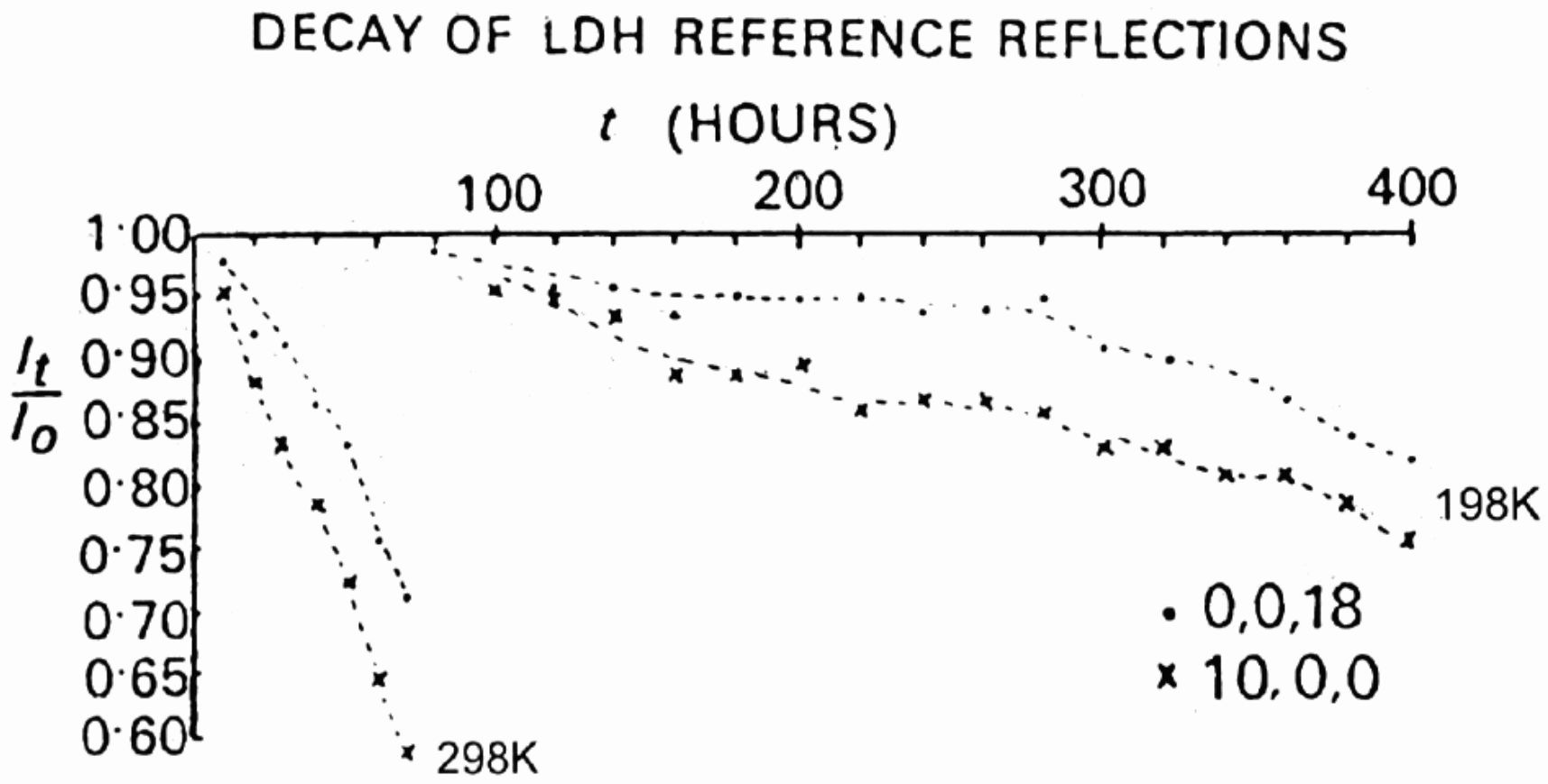


Protein: direct

Solvent: indirect

PRIMARY; inevitable, a fact of physics! Neutralise it?

SECONDARY, can we control it?

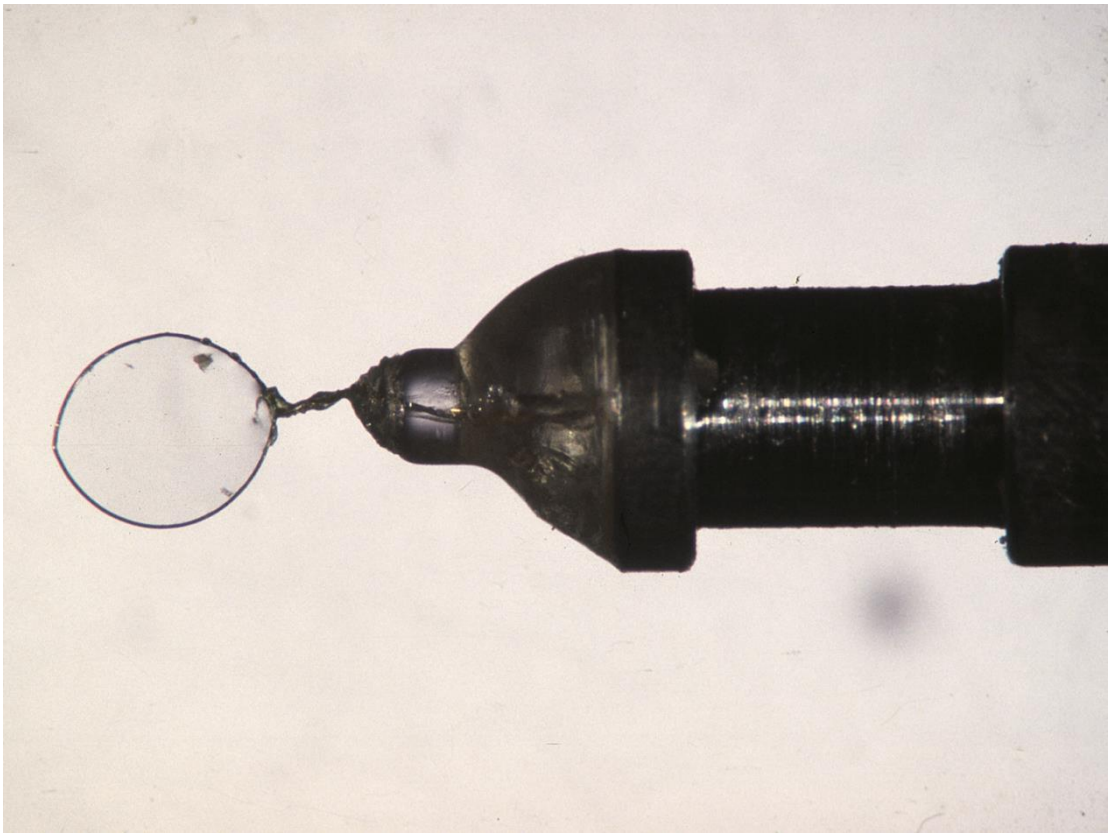


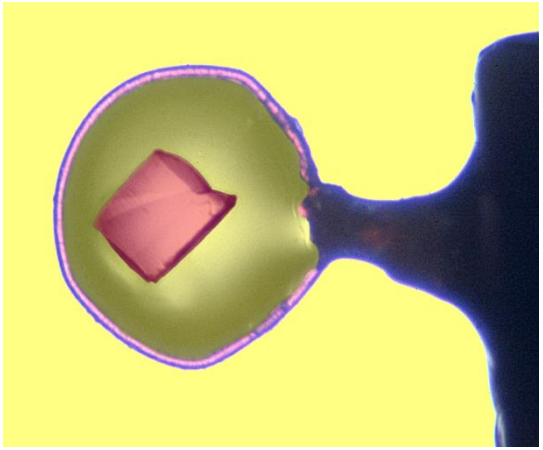
Haas and Rossmann 1970: lactate dehydrogenase

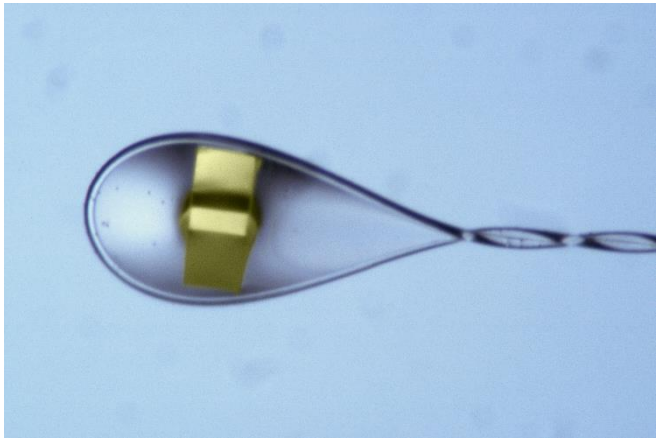
Acta Cryst B26, 998-1004.

ICE a major problem

Loop mounting: T-Y.Teng (1990) J.Appl.Cryst, 23, 387-391.
Used wire loops



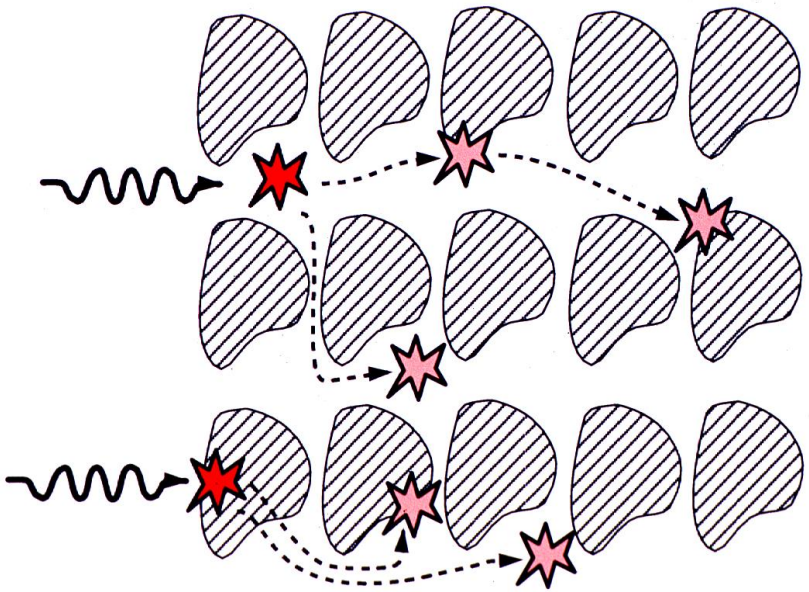


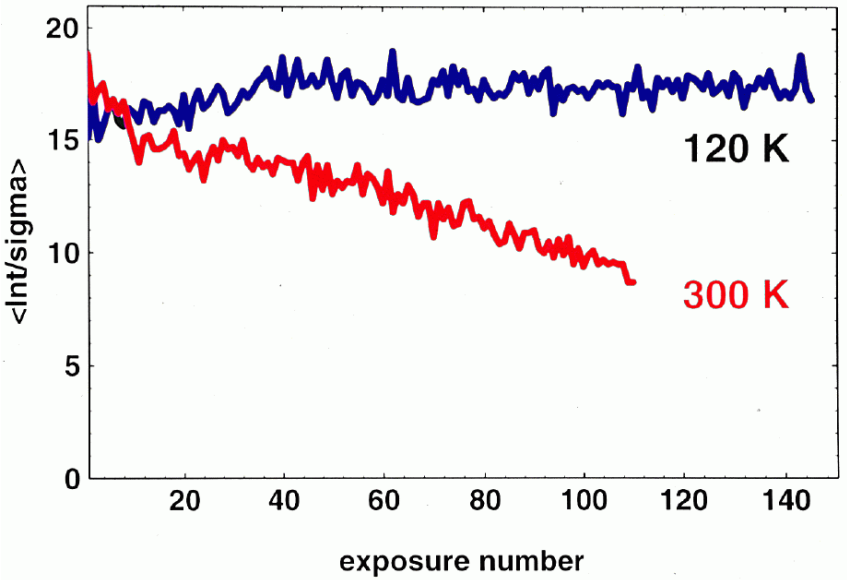


Also, a commercially available and easy to use cryostat (Cosier and Glazer 1986) made the technique accessible to many labs.

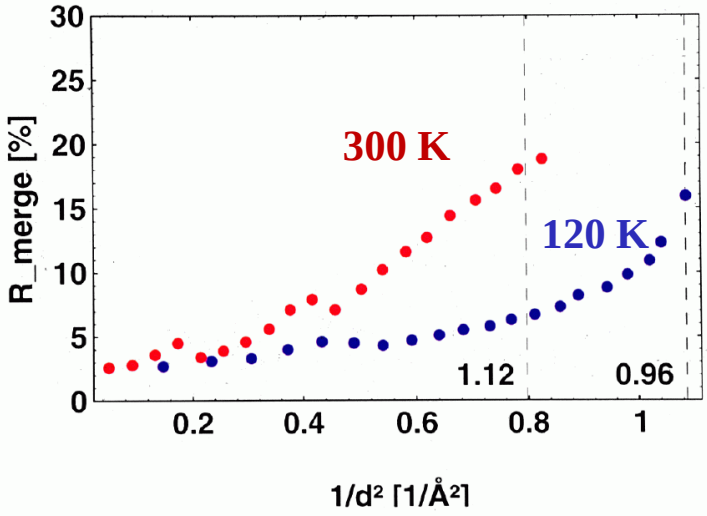
[Garman and Schneider, J.Appl.Cryst, (1997) **23**]

Radiation Damage





SP445: Data Quality [T.Schneider]



Significantly reduced at 100K: time factor of ~70

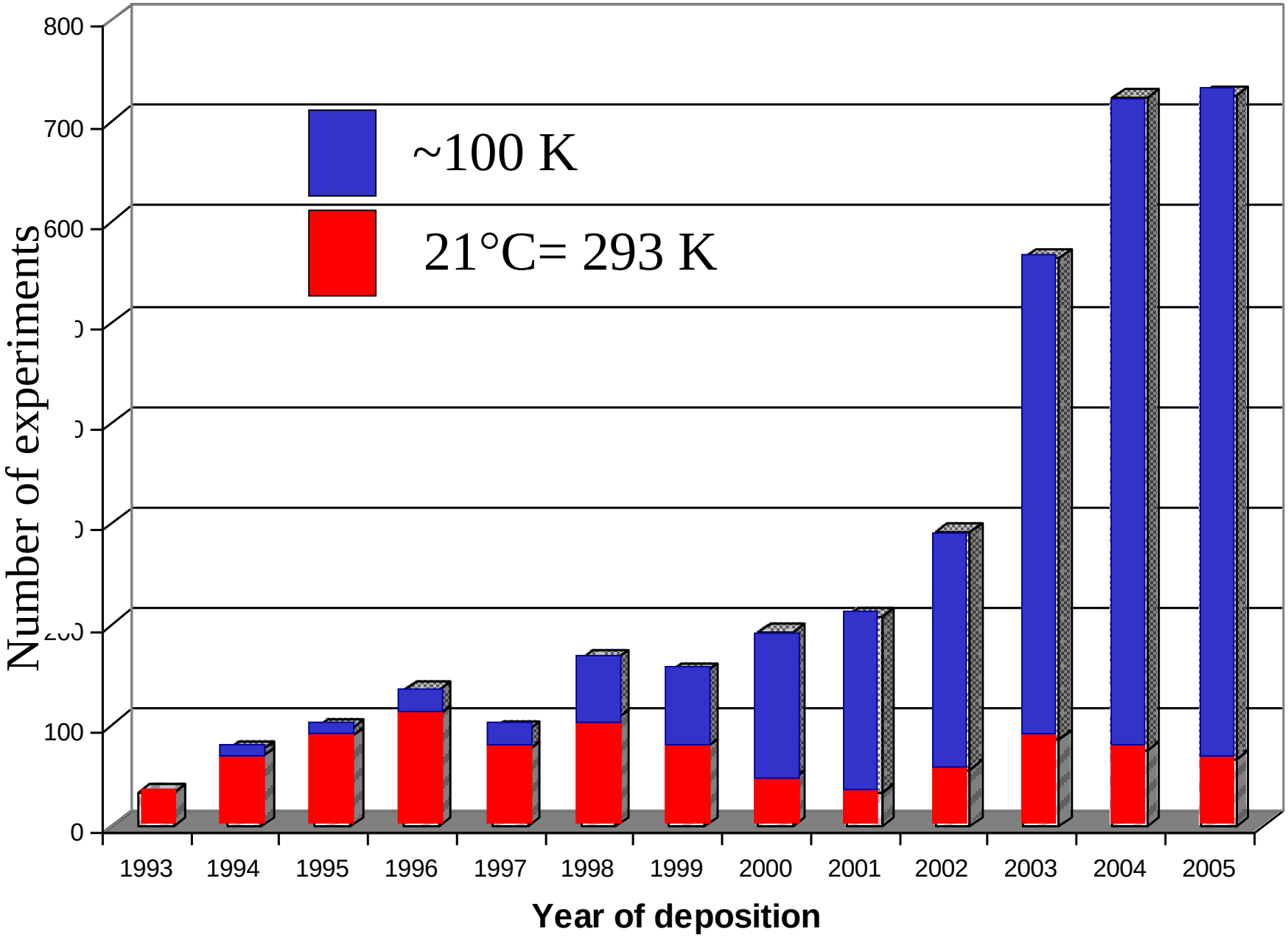
[Nave and Garman *JSR* (2005), **12**, 257-260].

PRIMARY; inevitable, a fact of physics!

SECONDARY, can we control it?

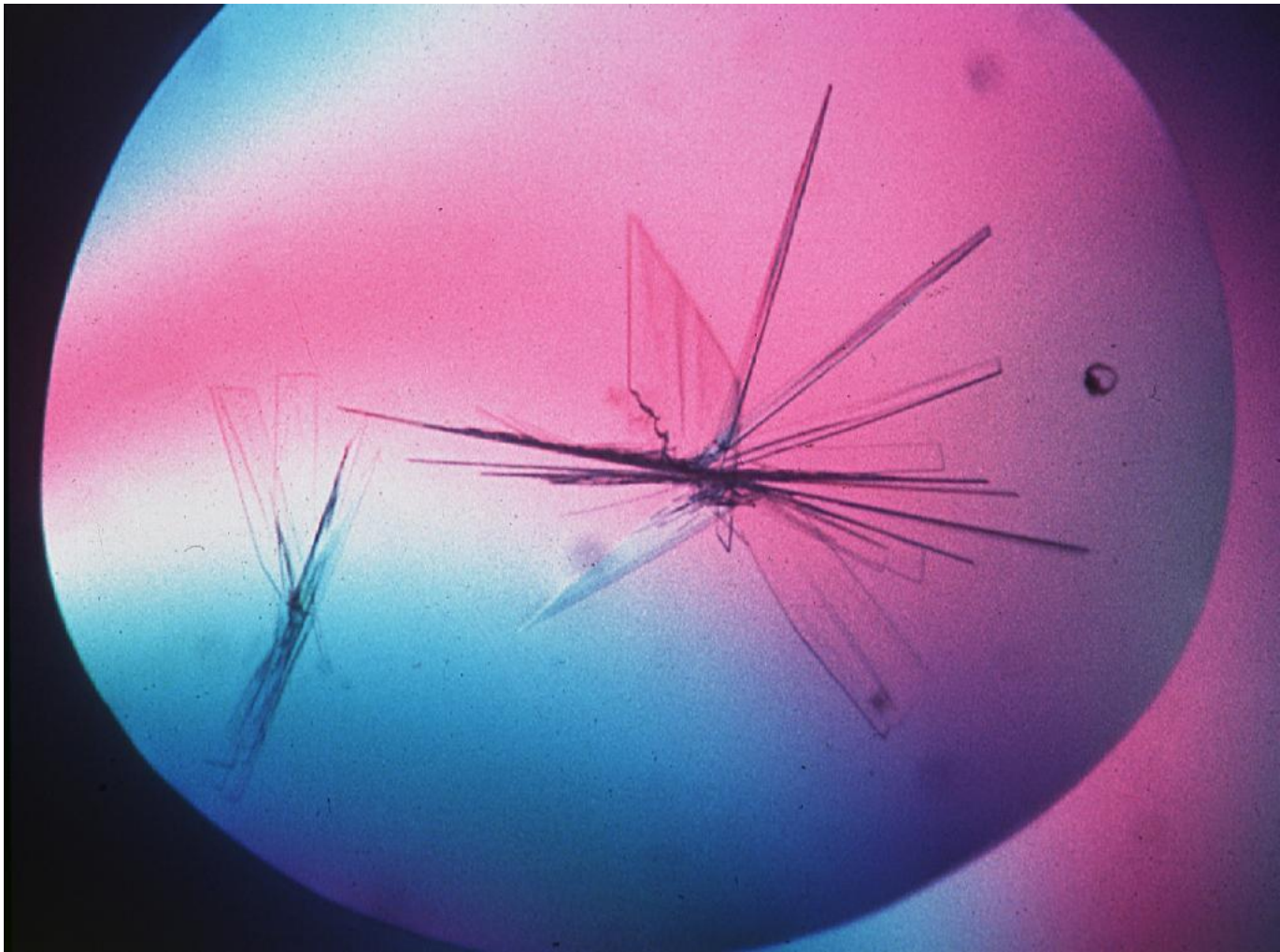
Proportions?

All experiments reported in Acta Crystallographica. D, 1993 – Dec 2005



[Garman, in Protein Crystallisation (Ed. T.Bergfors) 2009]

Loop mounting is a MUCH
gentler technique than capillary mounting.
e.g. cyclin A, 5μm x 100μm x 300μm



Other advantages:

- Usually get a whole data set from a single crystal $\Rightarrow$ higher QUALITY data.

  For MAD, the systematic errors are minimised by using only one crystal.
- Can harvest and store crystals while they are in peak condition.
- Small crystals and flat plates can be mounted easily.
- No secondary radiation damage during storage.
- New experiments are possible.

The Plan:

- Cryo techniques
  - Why cool? Radiation damage.
  - **Optimising cryoprotection.**
  - Testing at room temperature.
  - Storage and retrieval.
  - If nothing works…

Cryocooling: HOW?

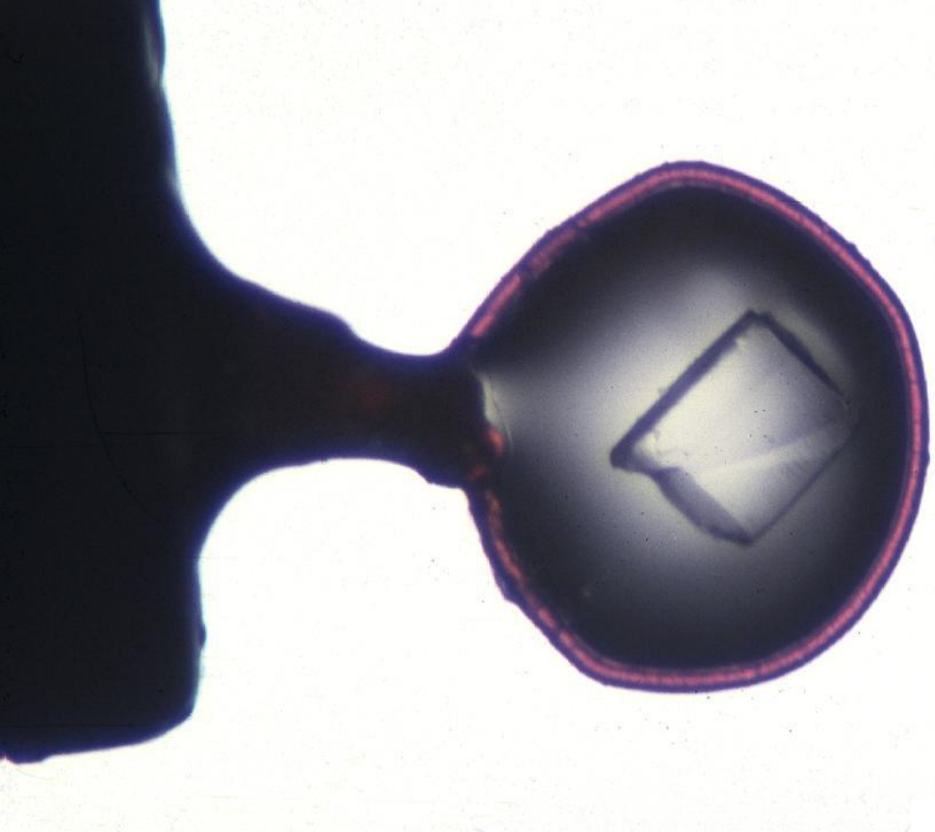
- Cool the crystal so fast that the water is vitrified and does not form crystalline ice. [Pure water: ~10^{-5} s for typical protein crystal sized drop]

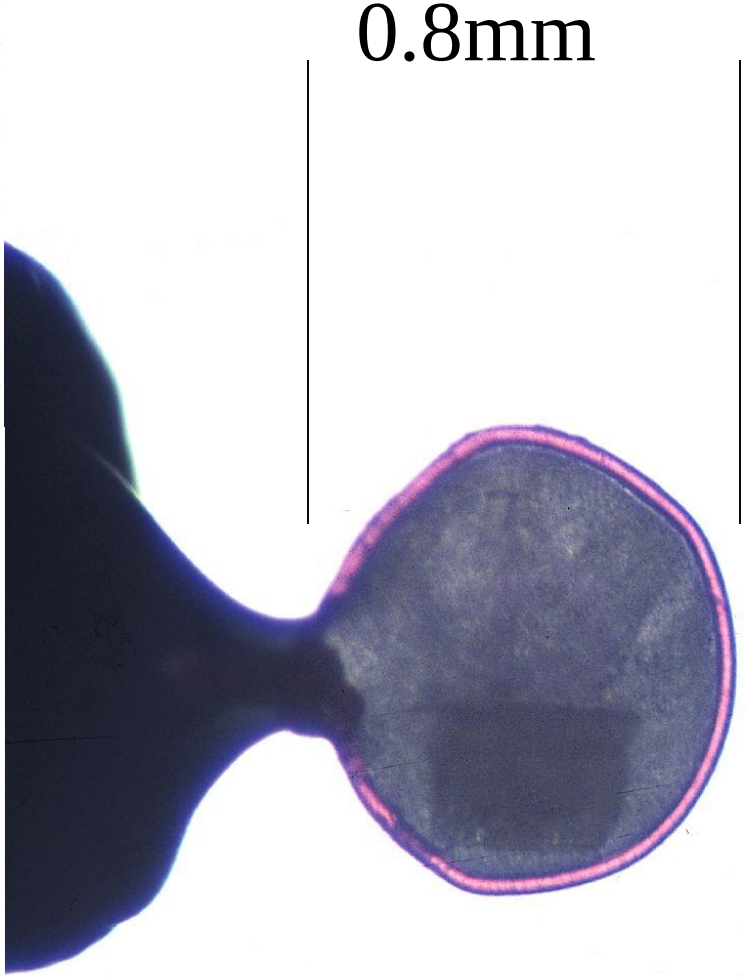
- Add `antifreeze' to increase time for cooling process. [1-2 s]

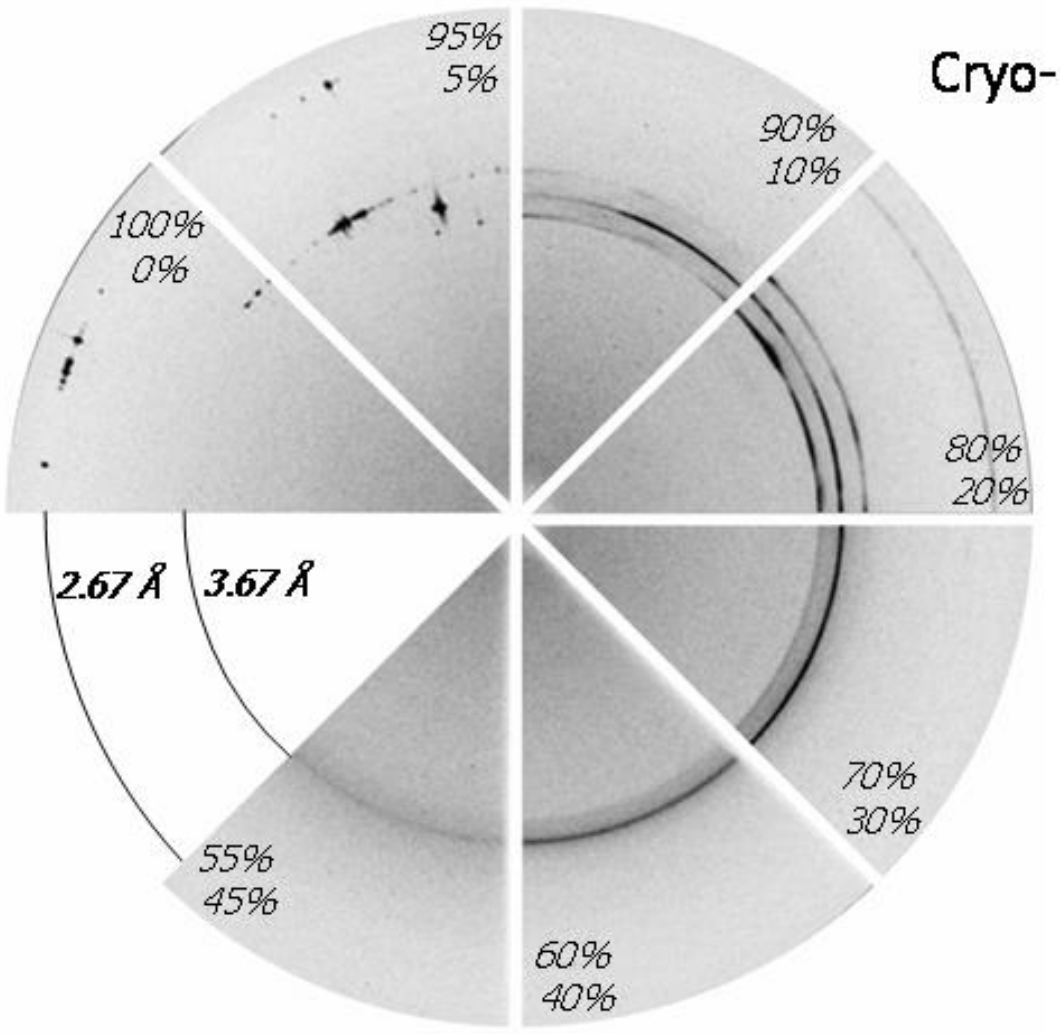
i.e. `cryoprotect'

N.B. WE DO **NOT** WANT TO 'FREEZE' the crystal!

- Collect data at around 100K [below 130K and absolutely below 155K [Weik, 2001]]




0.8mm



Cryo-buffer optimisation

hkl	d(Å)
100	3.9
002	3.67
101	3.44
102	2.67
110	2.25
103	2.07
200	1.95
112	1.92
201	1.88
202	1.72

- replace the water in mother liquor with cryo-agent, rather than diluting the mother liquor.
- test cryo-buffer alone in loop.

Cryo-buffers.

- PEG < 4K→ increase PEG, add small PEGs
- PEG ≥ 4K→ add small PEGs
- 2/3rds of cases → add 15 - 25% glycerol
- MPD → harvesting buffer, increase MPD concentration.
- Salt → add MPD and/or ethylene glycol or glycerol

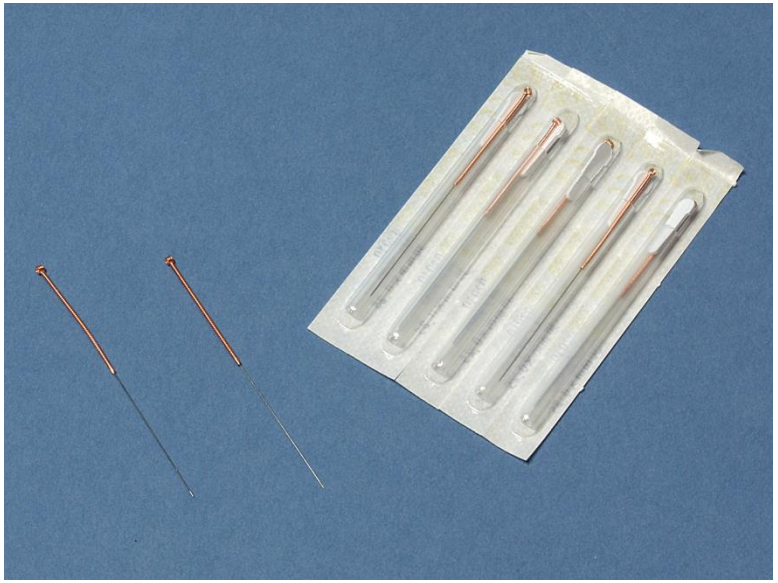
  → increase conc/add salt. Lithium salts good. [Rubinson et al, Acta D (2000), D56, 996-1001.]

  → Exchange salt. e.g. 100% 8M Na Formate.

N.B. Low salt needs > concentration of cryoprotectant than high salt.

Sugars, paratone N, combinations, +++

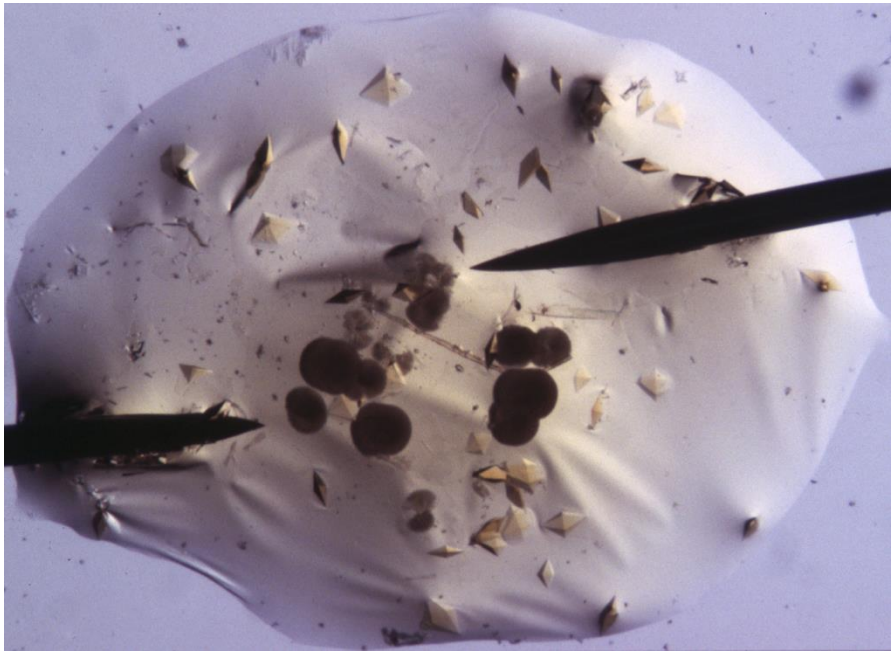
CRYSTAL MANIPULATION:



Acupuncture needles:
[free samples available from me]

- No loss of liquid
- Slightly flexible
- Fine
- Different sizes available.

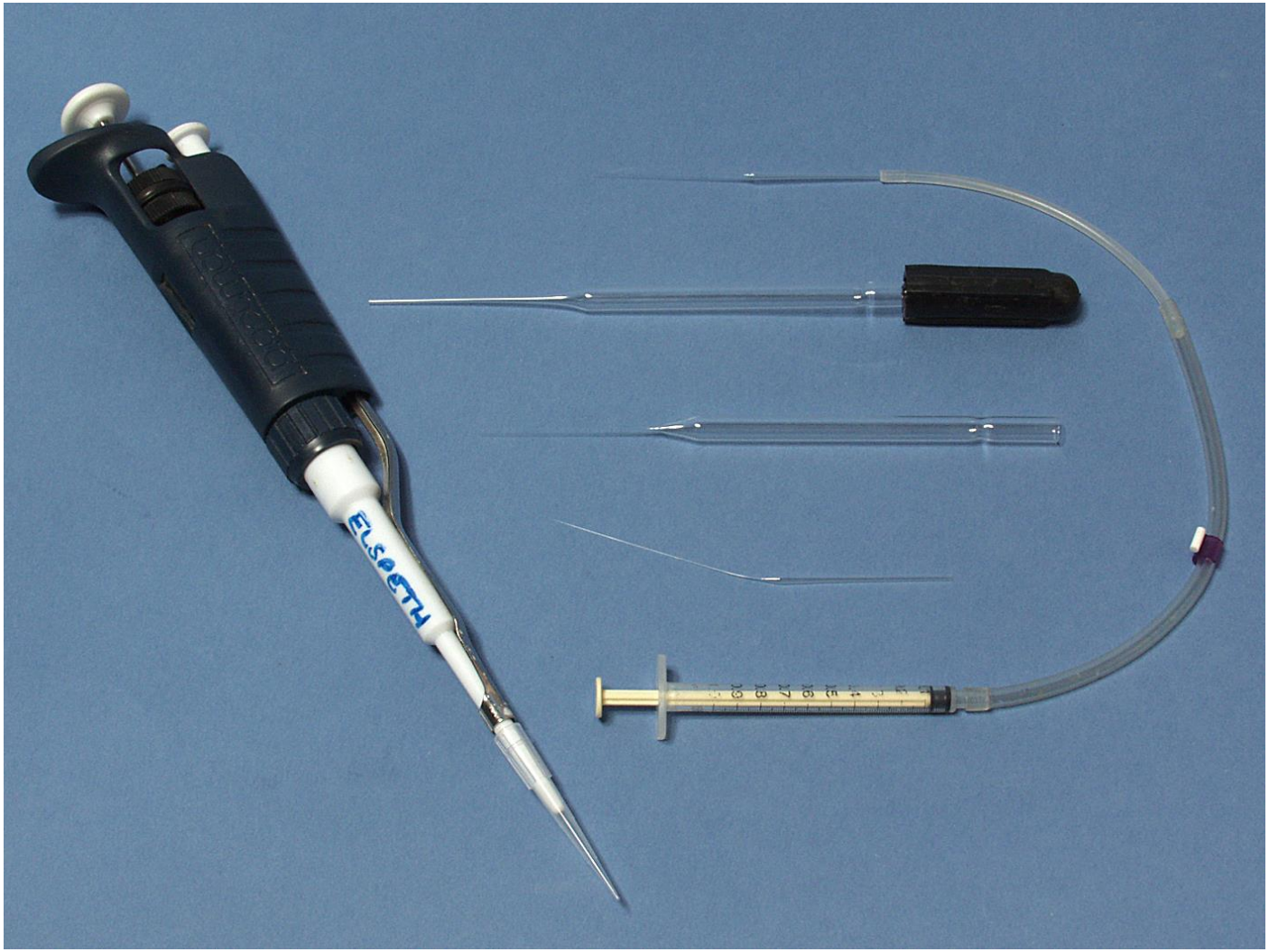
e.g. `Skin' on protein drop:

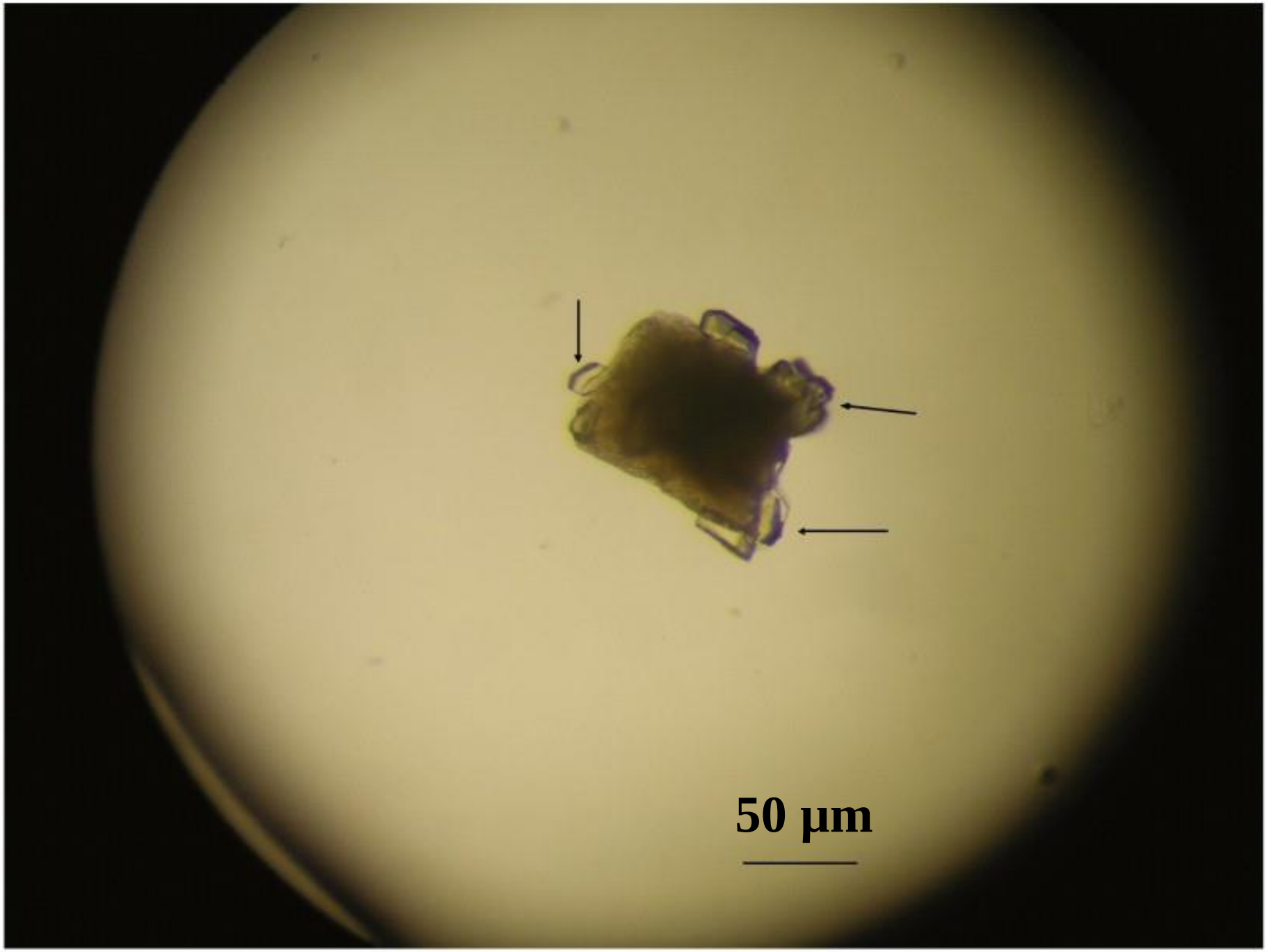


Gentle surgery
Skin doesn't diffract: just increases background

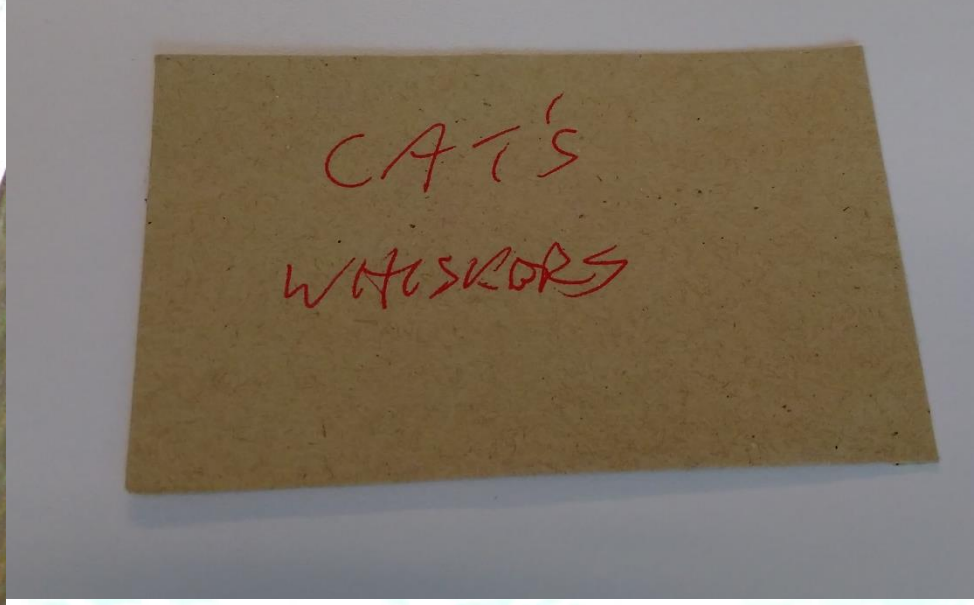
Cats' whiskers, horse eye lashes, horse tail hairs, etc etc

Manipulation of crystal from growth drop. A cryo-loop, or a syringe with flexible hose give much better control than a Gilson.




50 µm

Ellie
2002-2009



Megan, 1988-present

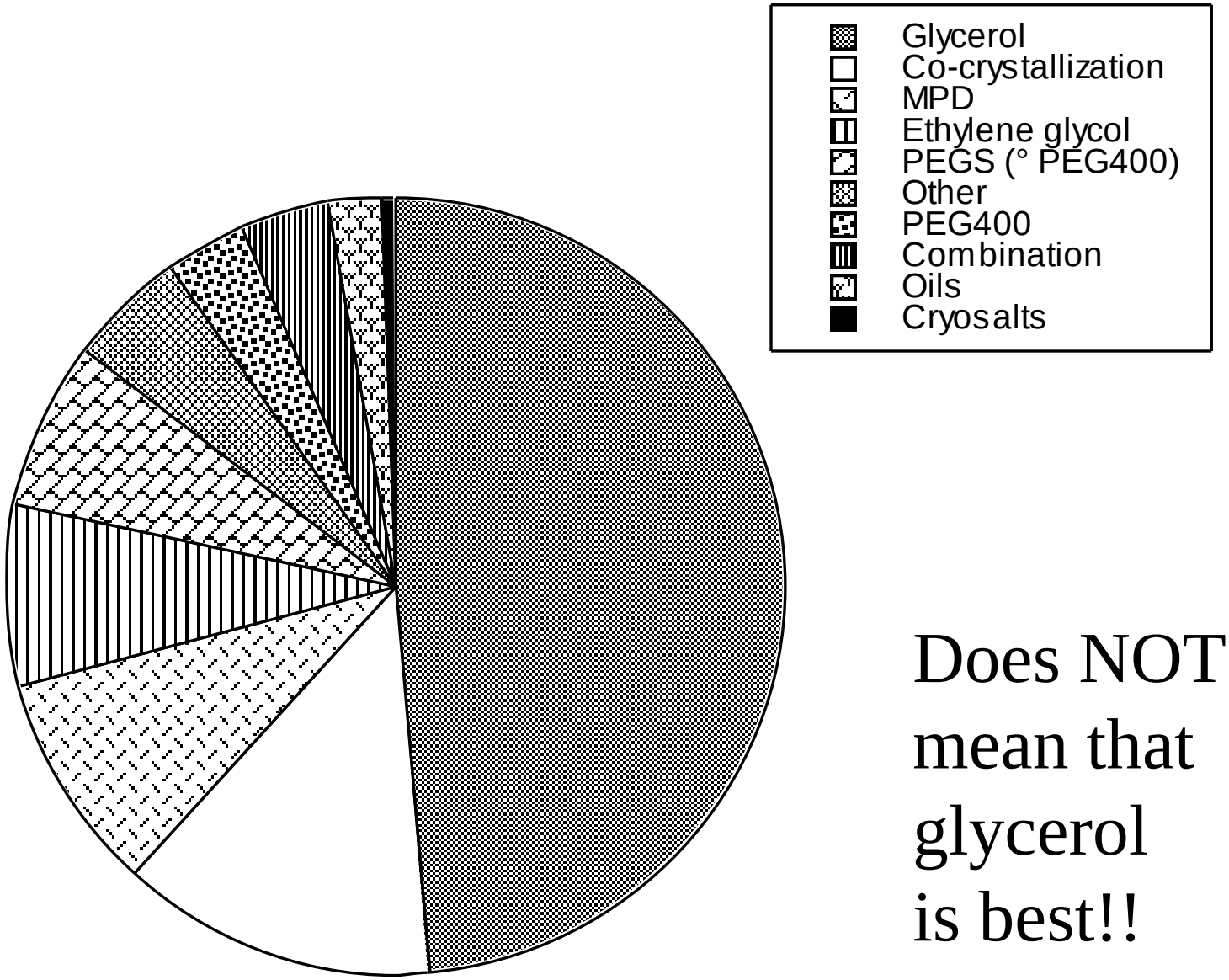
Illegible table of minimum concentrations of glycerol required to cryoprotect Hampton Screen I.

For emergency use only (done by dilution)

[Mitchell and Garman, J.Appl.Cryst. (1996) 29, 584
McFerrin and Snell J.Appl.Cryst (2002) 35, 538]

TABLE 1. Minimum concentration of glycerol to be added to solutions 1 to 50 of the Hampton Research Crystal Screen$^{(TM)}$ I Reagent Components to provide cryoprotection when frozen to 100K. It must be noted that glycerol was added to the Crystal Screen solutions resulting in a DILUTION of the original components. From Garman and Mitchell J. Appl. Cryst.(1996) 29, 584-587

Solution number, SALT	BUFFER	PRECIPITANT	GLYCEROL CONC. v/v
1. 0.02 M Ca Chloride	0.1 M Na Acetate pH 4.6	30% v/v 2-methyl-2,4-pentanediol	0
2. None	None	0.4 M K, Na Tartrate	35
3. None	None	0.4 M NH4 Phosphate	35
4. None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Sulphate	25
5. 0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	30% v/v 2-methyl-2,4-pentanediol	0
6. 0.2 M Mg Chloride	0.1 M Tris HCl pH 8.5	30% w/v PEG 4000	20
7. None	0.1 M Na Cacodylate pH 6.5	1.4 M Na Acetate	30
8. 0.2 M Na Citrate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-propanol	30
9. 0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% w/v PEG 4000	15
10. 0.2 M NH4 Acetate	0.1 M Na Acetate pH 4.6	30% w/v PEG 4000	15
11. None	0.1 M Na Citrate pH 5.6	1.0 M NH4 Phosphate	30
12. 0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v 2-propanol	10
13. 0.2 M Na Citrate	0.1 M Tris HCl pH 8.5	30% v/v PEG 400	0
14. 0.2 M Ca Chloride	0.1 M Na Hepes pH 7.5	28% v/v PEG 400	5
15. 0.2 M NH4 Sulphate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000	15
16. None	0.1 M Na Hepes pH 7.5	1.5 M Li Sulphate	25
17. 0.2 M Li Sulphate	0.1 M Tris HCl pH 8.5	30% PEG 4000	15
18. 0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	20% PEG 8000	20
19. 0.2 M NH4 Acetate	0.1 M Tris HCl pH 8.5	30% v/v 2-propanol	20
20. 0.2 M NH4 Sulphate	0.1 M Na Acetate pH 4.6	25% w/v PEG 4000	20
21. 0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-methyl-2,4-pentanediol	0
22. 0.2 M Na Acetate	0.1 M Tris HCl pH 8.5	30% w/v PEG 4000	15
23. 0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v PEG 400	0
24. 0.2 Ca Chloride	0.1 M Na Acetate pH 4.6	20% v/v 2-propanol	30
25. None	0.1 M Imidazole pH 6.5	1.0 M Na Acetate	30
26. 0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% v/v 2-methyl-2,4-pentanediol	0
27. 0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	20 % v/v 2-propanol	30
28. 0.2 M Na Acetate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000	15
29. None	0.1 M Na Hepes pH 7.5	0.8 M K,Na Tartrate	35
30. 0.2 M NH4 Sulphate	None	30% w/v PEG 8000	15
31. 0.2 M NH4 Sulphate	None	30% w/v PEG 4000	15
32. None	None	2.0 M NH4 Sulphate	25
33. None	None	4.0 M Na Formate	10
34. None	0.1 M Na Acetate pH 4.6	2.0 M Na Formate	30
35. None	0.1 M Na Hepes pH 7.5	1.6 M Na, K Phosphate	25
36. None	0.1 M Tris HCl pH 8.5	8% w/v PEG 8000	35
37. None	0.1 M Na Acetate pH 4.6	8% w/v PEG 4000	30
38. None	0.1 M Na Hepes pH 7.5	1.4 M Na Citrate	10
39. None	0.1 M Na Hepes pH 7.5	2% v/v PEG 400 and 2.0 M NH4 Sulphate	15
40. None	0.1 M Na Citrate pH 5.6	20% v/v 2-propanol and 20% w/v PEG 4000	5
41. None	0.1 M Na Hepes pH 7.5	10% v/v 2-propanol and 20% w/v PEG 4000	15
42. 0.05 M K Phosphate	None	20% w/v PEG 8000	20
43. None	None	30% v/v PEG 1500	20
44. None	None	0.2 M Mg Formate	50
45. 0.2 M Zn Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000	20
46. 0.2 M Ca Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000	20
47. None	0.1 M Na Acetate pH 4.6	2.0 M NH4 Sulphate	20
48. None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Phosphate	20
49. 1.0 M Li Sulphate	None	2% w/v PEG 8000	20
50. 0.5 M Li Sulphate	None	15% w/v PEG 8000	20



Does NOT mean that glycerol is best!!

Acta D structures 2001. [Garman and Doublié, Methods in Enzymology 2003, 368, 188.]

Transfer of crystal into cryobuffer:

1) Dialysis of cryoprotectant.

[Fernandez *et al*, JAPC (2000) 33, 168-171]

2) Co-crystallisation with cryoprotectant agent:
glycerol is already known to help in some cases

[Sousa, Acta Cryst D51 (1995) 271-277.]

3) Rapid transfer
   - Straight into final concentration for up to 5 mins
   - can just `sweep through'- 0.5 sec
   - Sequential soaks in increasing concentrations.

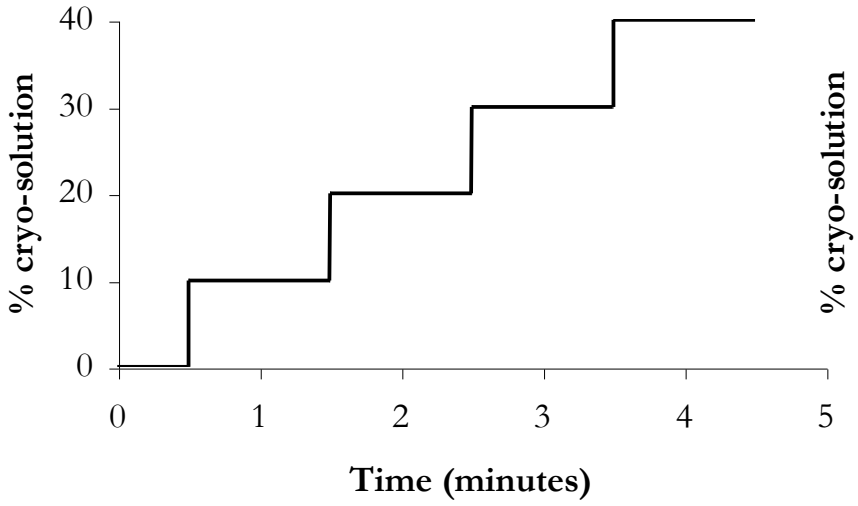
WANT TO MINIMISE HANDLING, as handling can increase the mosaic spread. i.e. 2) is BEST:….

SOLUBILITY versus OSMOTIC shock

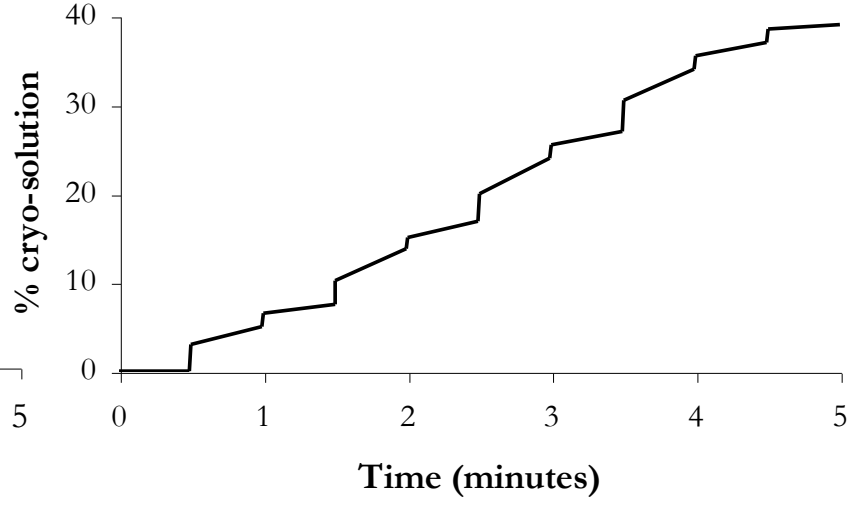
Crystal transfer optimisation:

- balance osmotic shock and attack of crystal surface
- serial transfers: minimise handling and dehydration

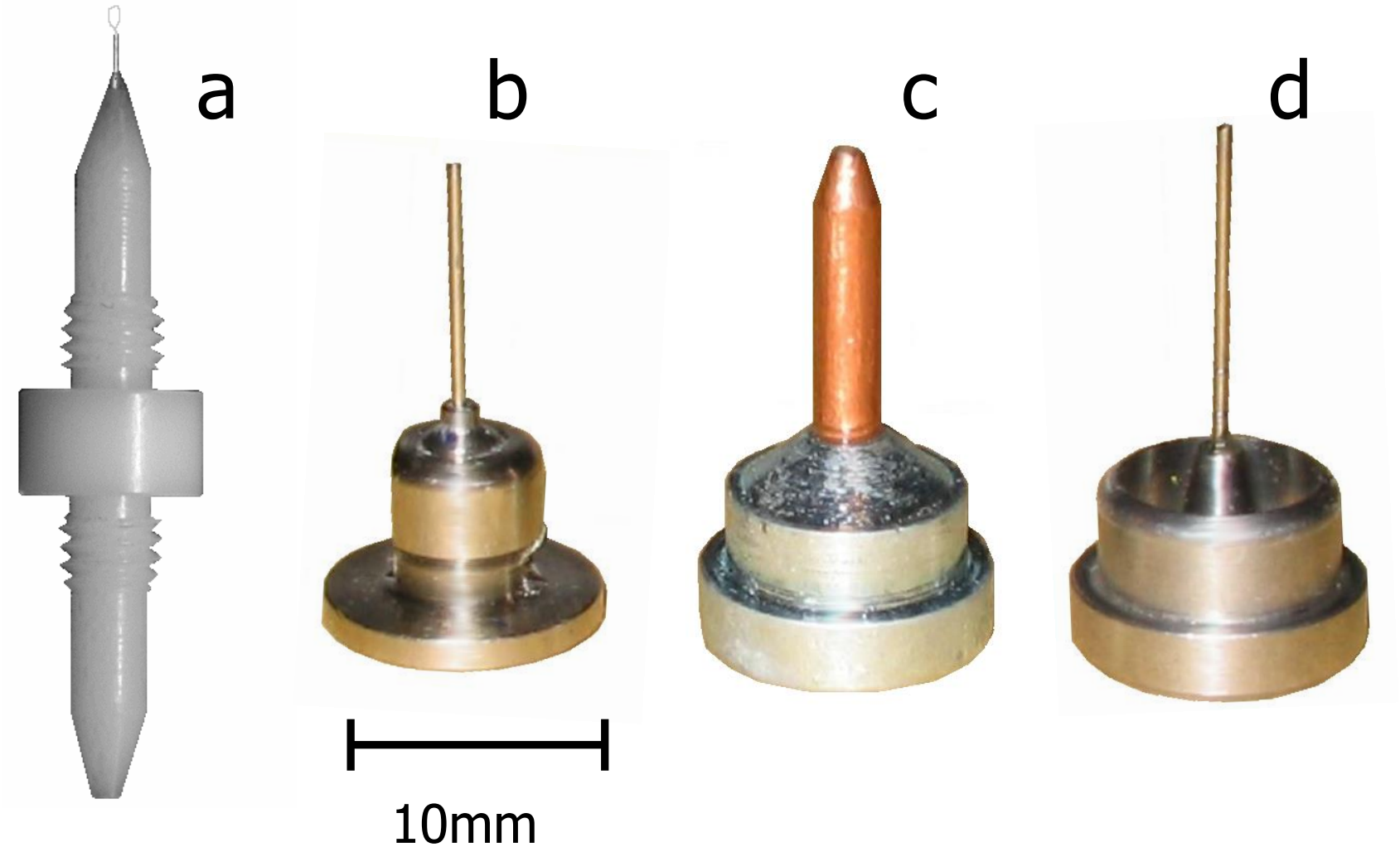
a) Move crystal between solutions



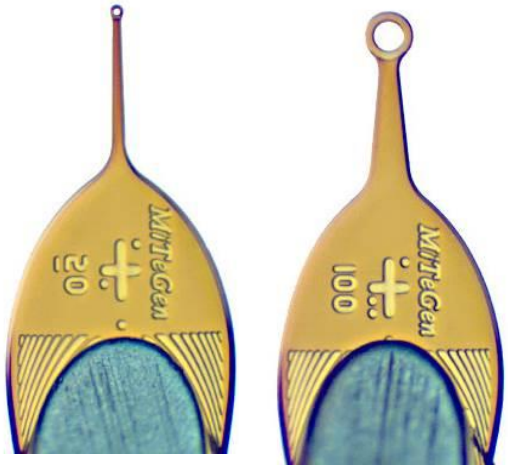
b) Solution pipetted onto crystal



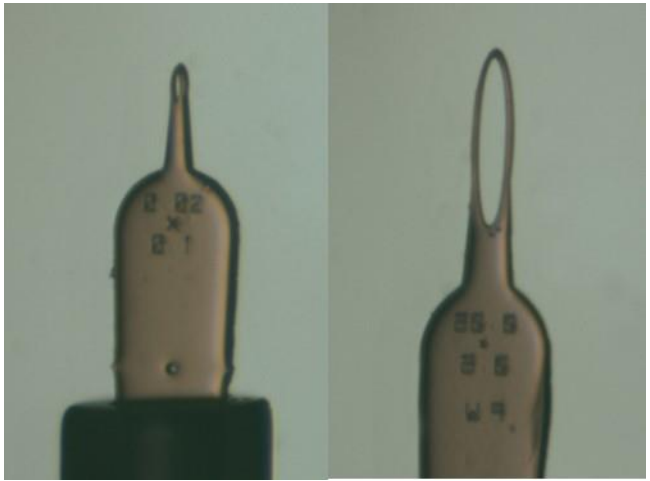
Hardware development: standardisation…



Litho-loops,
etched mylar,
ActiLoop polymer
Molecular Dimensions



Rayon loops
Hampton Research

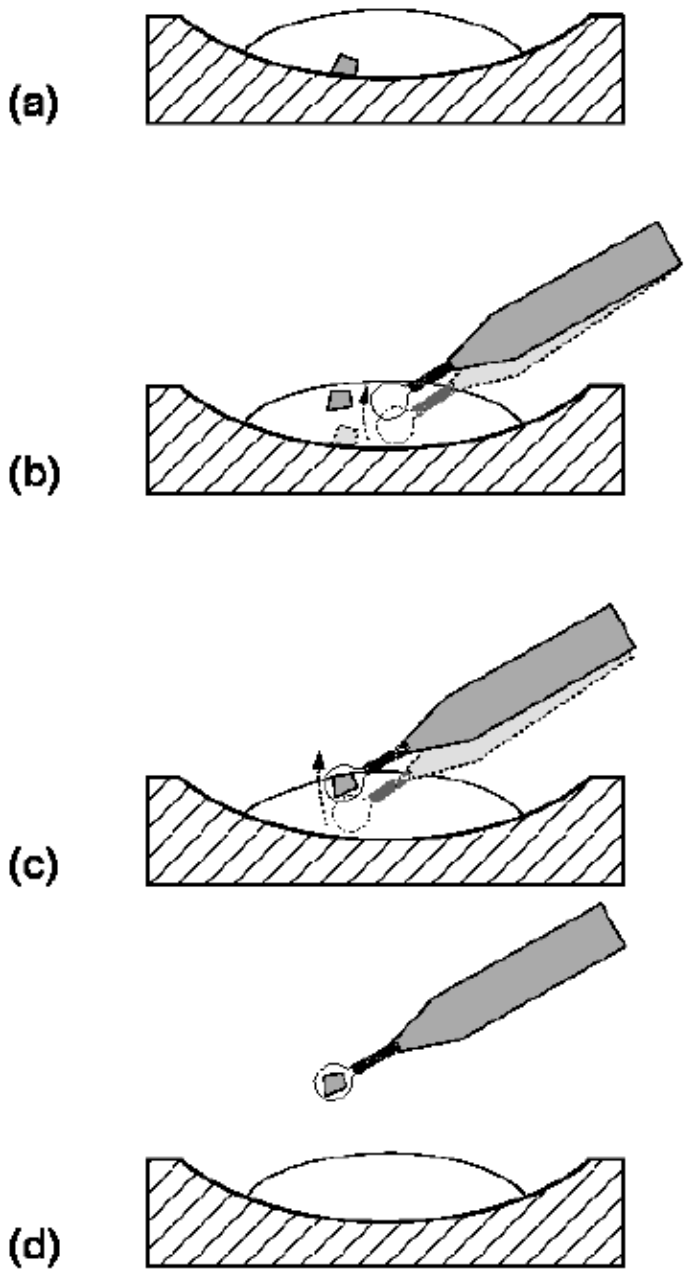


Micro-mounts,
microfabricated
polyimide film. **MiTeGen**
[Thorne *et al* (2003), JAPC 36, 1455.]

Fishing:

- minimise handling
- minimise liquid round crystal
- fish near cryogen
- loop perpendicular to liquid

N.B. Acupuncture needles.
Salt crystals in loop.



Flash cool into cryogen:

- Straight into liquid nitrogen:

  have a standard pin length AND

  blow/flap away surface cold nitrogen from FULL Dewar.

- Stream cool into nitrogen gas stream held at around 100K: pre-align pin.

  have a standard pin length!

PRACTICE!!!

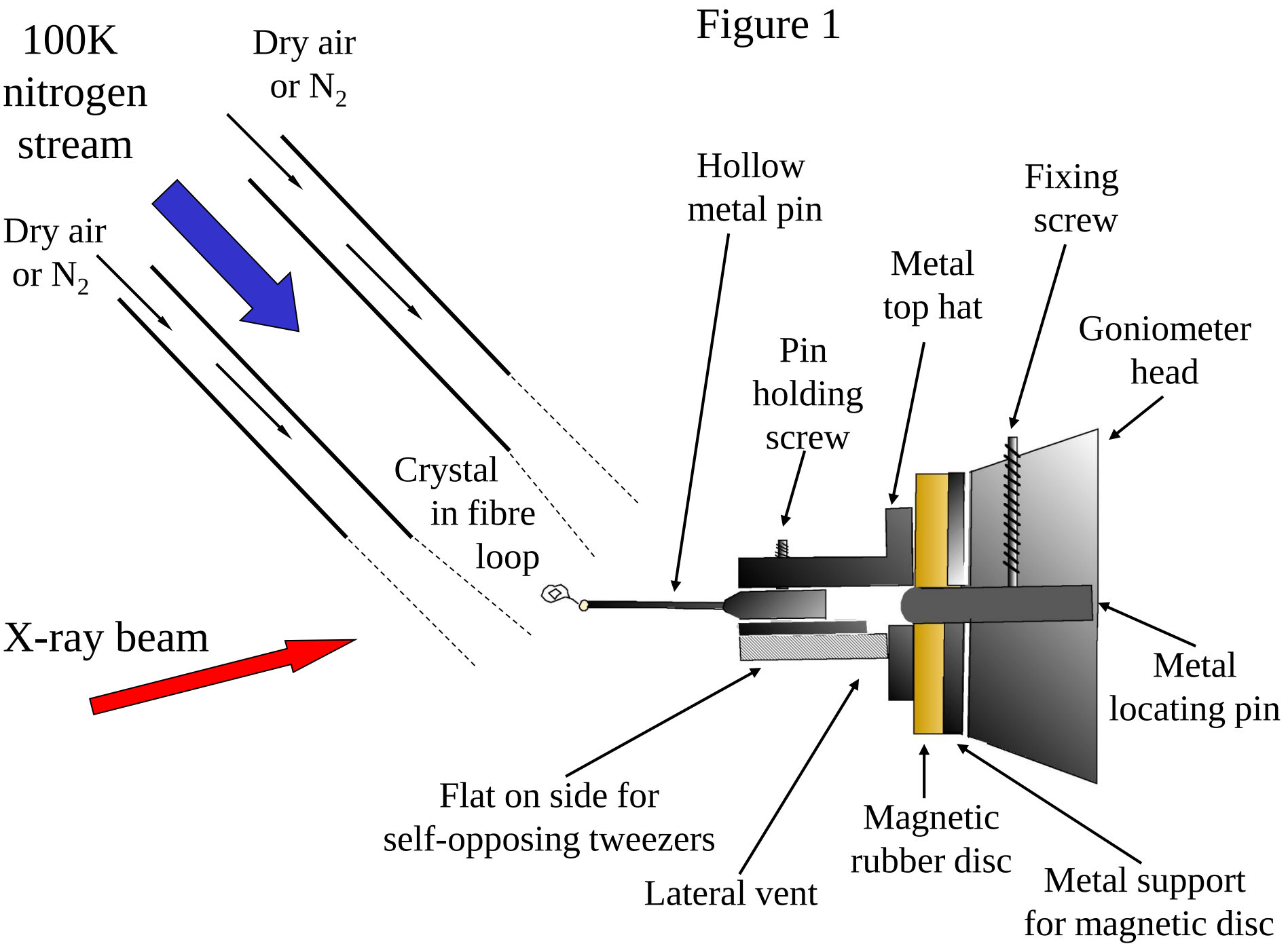
(but not with your most precious crystals)

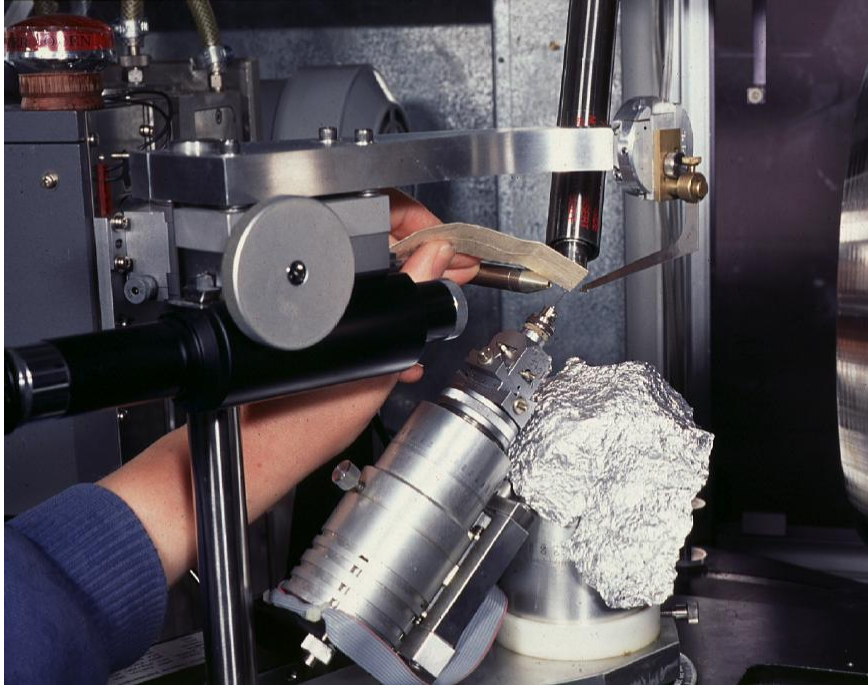
Humidity device to avoid dehydration during mounting

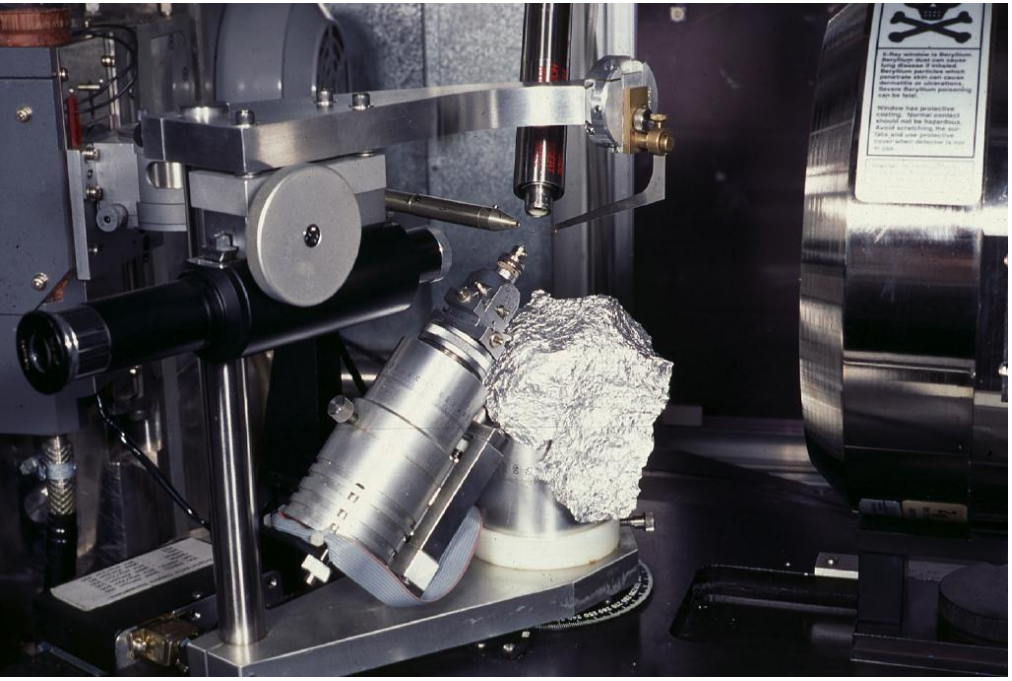
Especially effective for those mother liquors which phase separate when crystallisation drop is opened.

[Farley et al., Juers Acata Cryst D (2014) 70:2111-24]

Figure 1


100K nitrogen stream
Dry air or N_2
Dry air or N_2
Hollow metal pin
Fixing screw
Metal top hat
Goniometer head
Pin holding screw
Crystal in fibre loop
X-ray beam
Metal locating pin
Flat on side for self-opposing tweezers
Lateral vent
Magnetic rubber disc
Metal support for magnetic disc





- Pre-centre loop.
- Speed of transfer from drop
- Block stream

[avoid dehydration]

- Speed of cooling

Why bother to cover the nitrogen stream?

- Avoid crystal being dehydrated by dry nitrogen/air.
- You may wave crystal in and out of stream while cooling it: slow cooling will give ice.
- Want to cool it FAST. Much easier to do that if you whip the cover away once crystal is in position.

DIFFRACTION?

- NO …

  Does the crystal diffract at room temperature??

NO	YES
↓	↓
Back to crystallisation trials	Change cryo-protocol

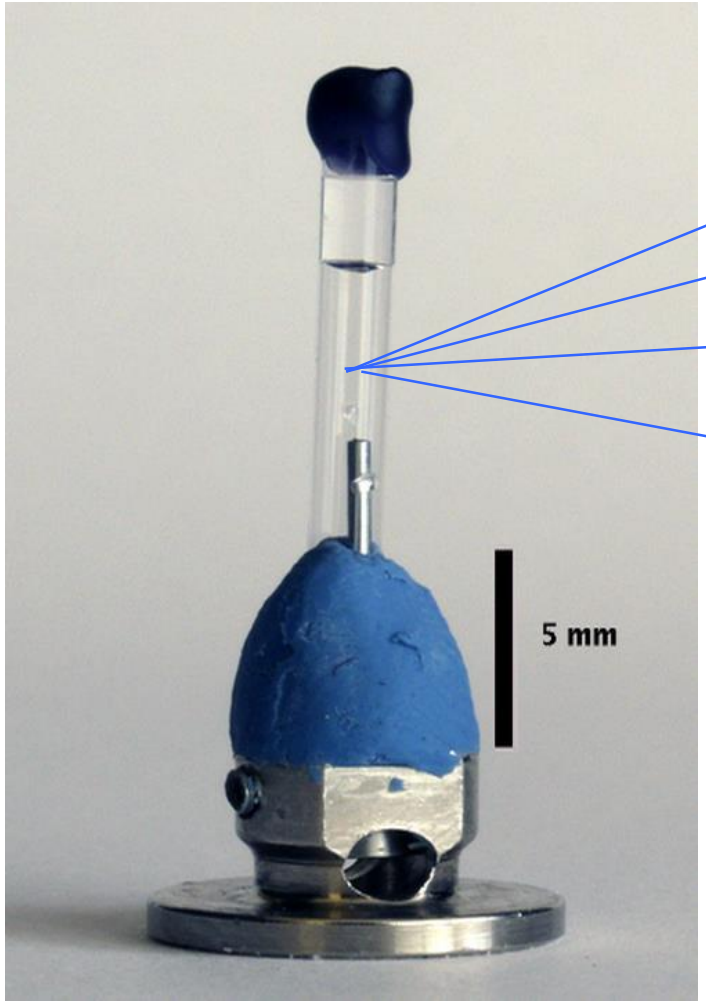
- YES… then take data and/or store it in a Dewar.

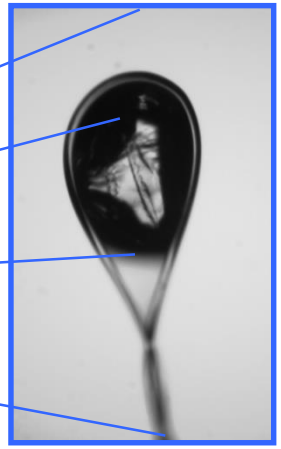
The Plan:

- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - **Testing at room temperature.**
  - Storage and retrieval.
  - If nothing works…

(Much) easier RT mounting method

Allows protein crystals to be mounted at room temperature in loop.



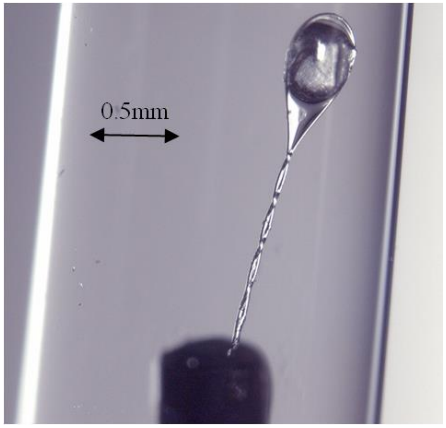


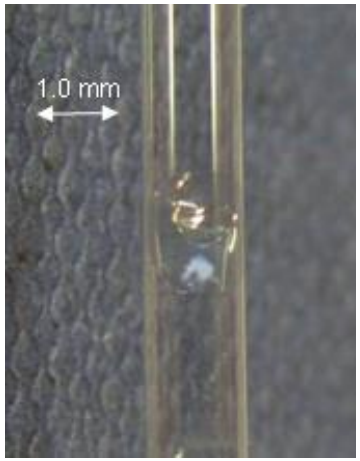
Capillary prevents drop drying out at room temperature.

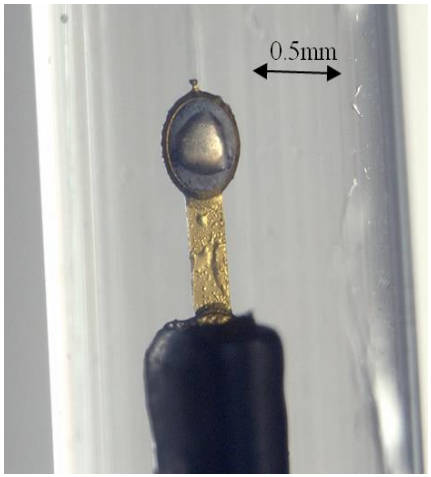
Also eliminates crystal manipulation between room temperature and cryo-cooled datasets.

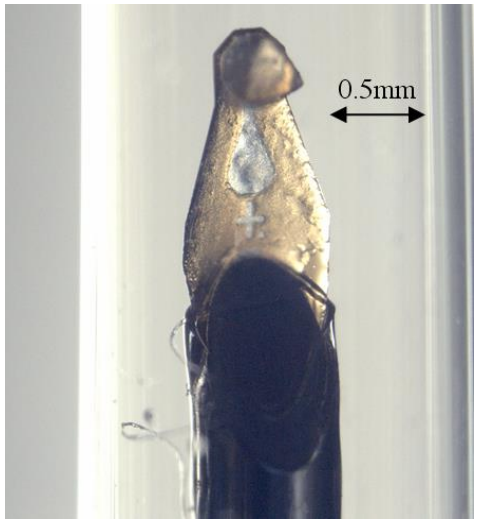
Skrzypczak-Jankun *et al* Acta. Cryst. (1996). **D52**, 959-965

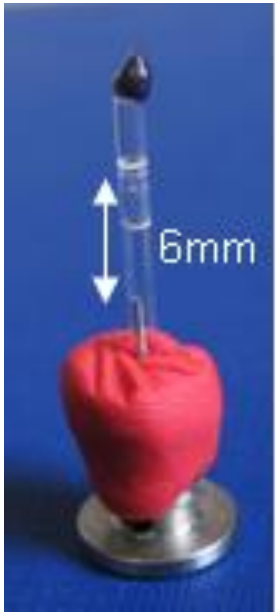
Room temperature crystal mounting methods

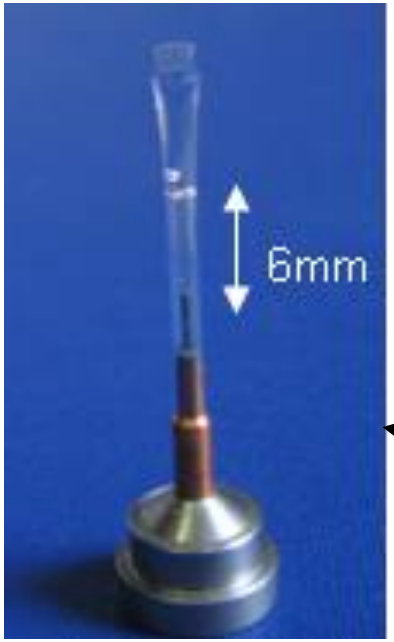




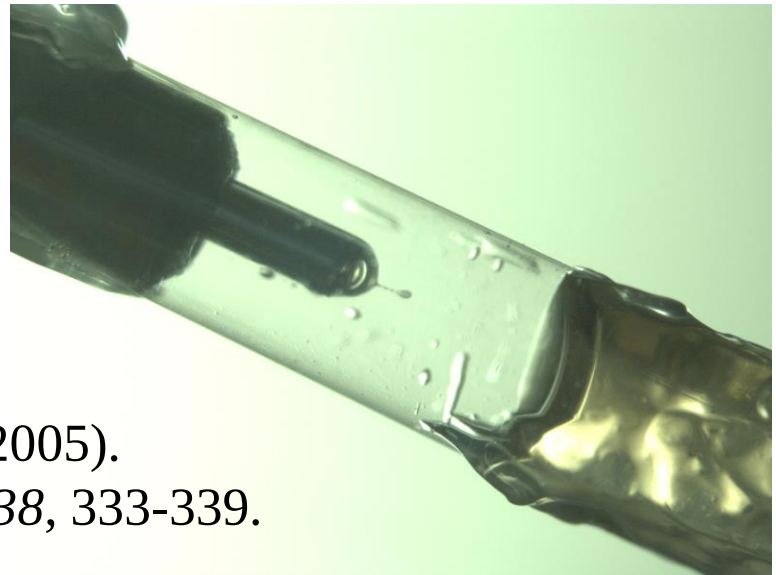








MiTeGen



Kalinin *et al*. (2005).
J. App. Cryst. *38*, 333-339.

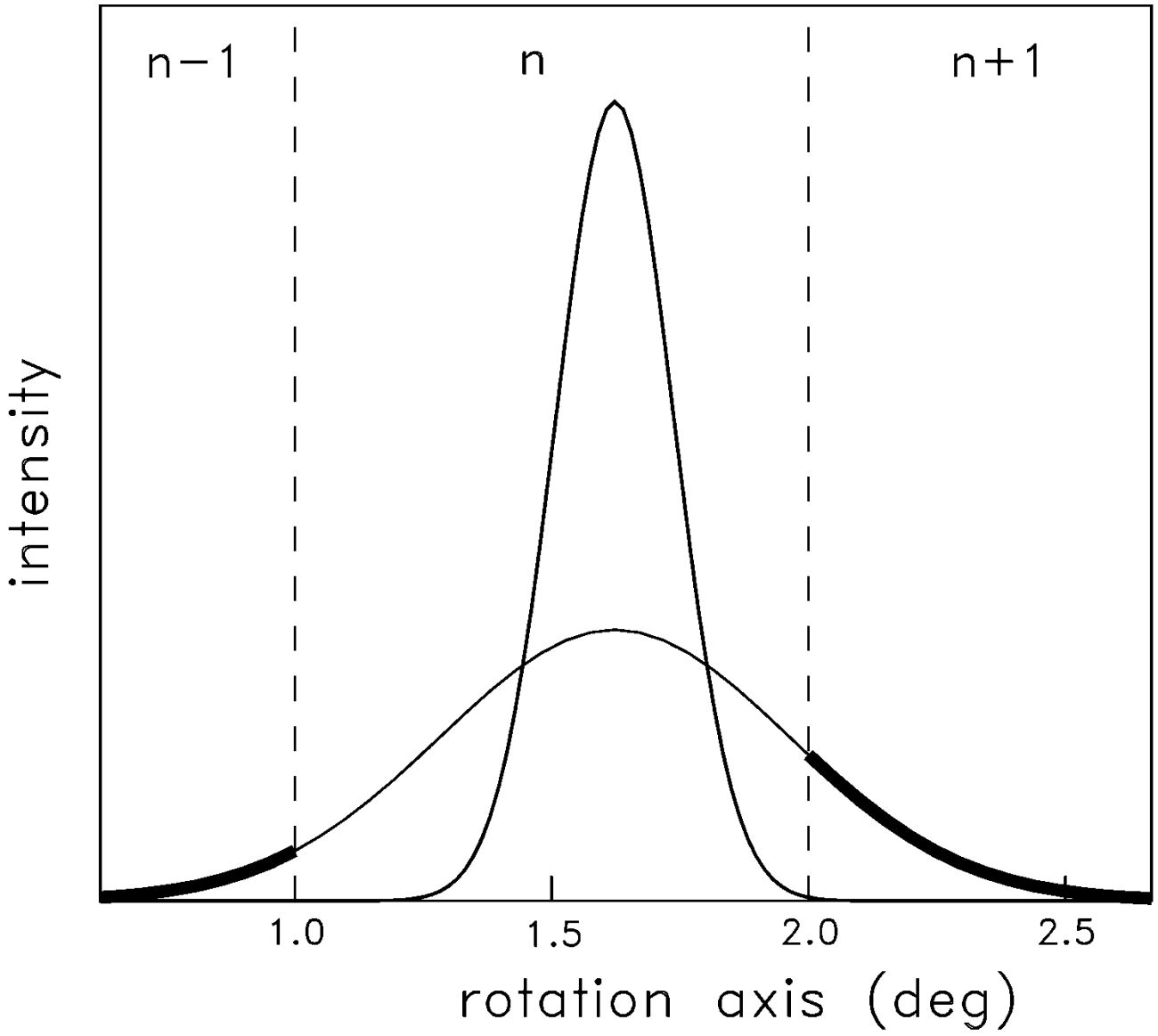
MOSAICITY:

- Want to minimise mosaicity but it often increases.
- Are cryoconditions optimised?
  - solutions
  - transfer
- Speed of transfer to cryogen
- Speed of cooling
- Size of crystal:

Should be able to reproduce room temp mosaicity [need to know it!].

Minimise mosaic spread to optimise data quality.

Should be able to reproduce room temp mosaicity [need to know it!].



Cryo-protocol Optimisation:
maximising resolution, [minimise mosaicity]

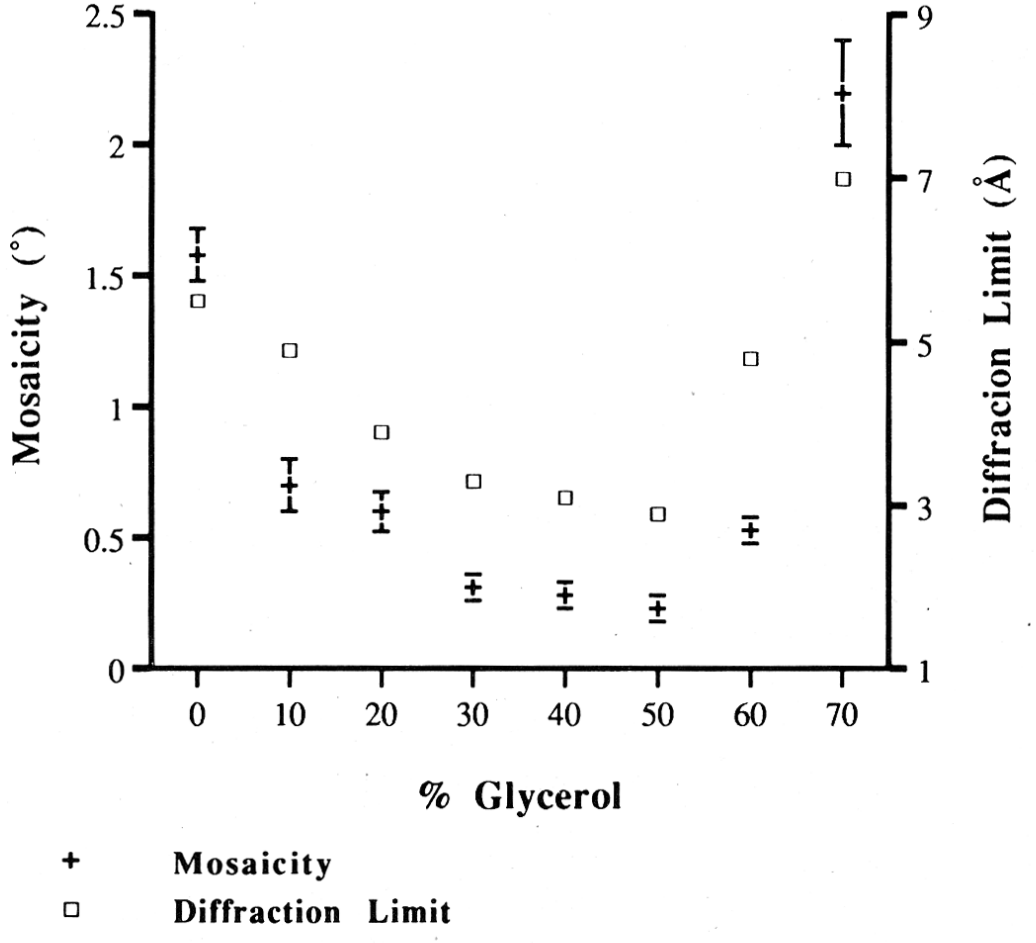


Fig. 2. Variation of mosaicity and diffraction limit of GPb crystals with percentage of glycerol in the buffer. The error bars represent statistical counting errors only.

Mitchell and Garman, J.Appl.Cryst. (1994) 27, 1070-1074

The Plan:

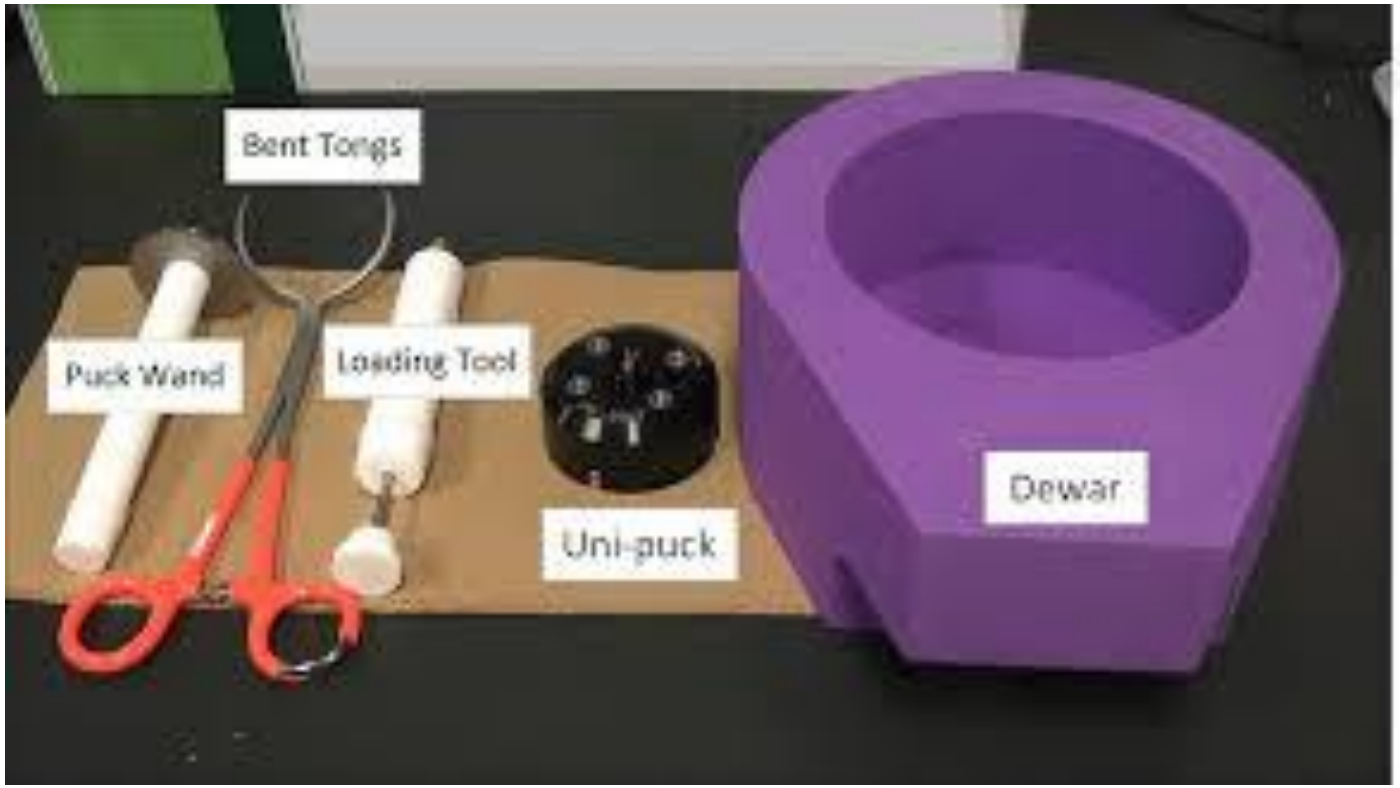
- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - Testing at room temperature.
  - **Storage and retrieval**
  - If nothing works…

Crystal
storage
and
retrieval

Farside
copyrighted cartoon

Hyperquenching: want a FAST cool

Keep Dewar full or remove gas layer from above liquid.



[Warkentin et al., Thorne, J. Appl. Cryst. (2006) 39, 805]

STORAGE:

- Label the vials beforehand.
- Label the canes.
- Allow transport Dewars to dry out after each trip.

Dewar drying rack, Dept Biochemistry, Oxford, UK

Excellent Dewar testing protocol

http://smb.slac.stanford.edu/facilities/hardware/cryotools/shipping-dewar-testing.html

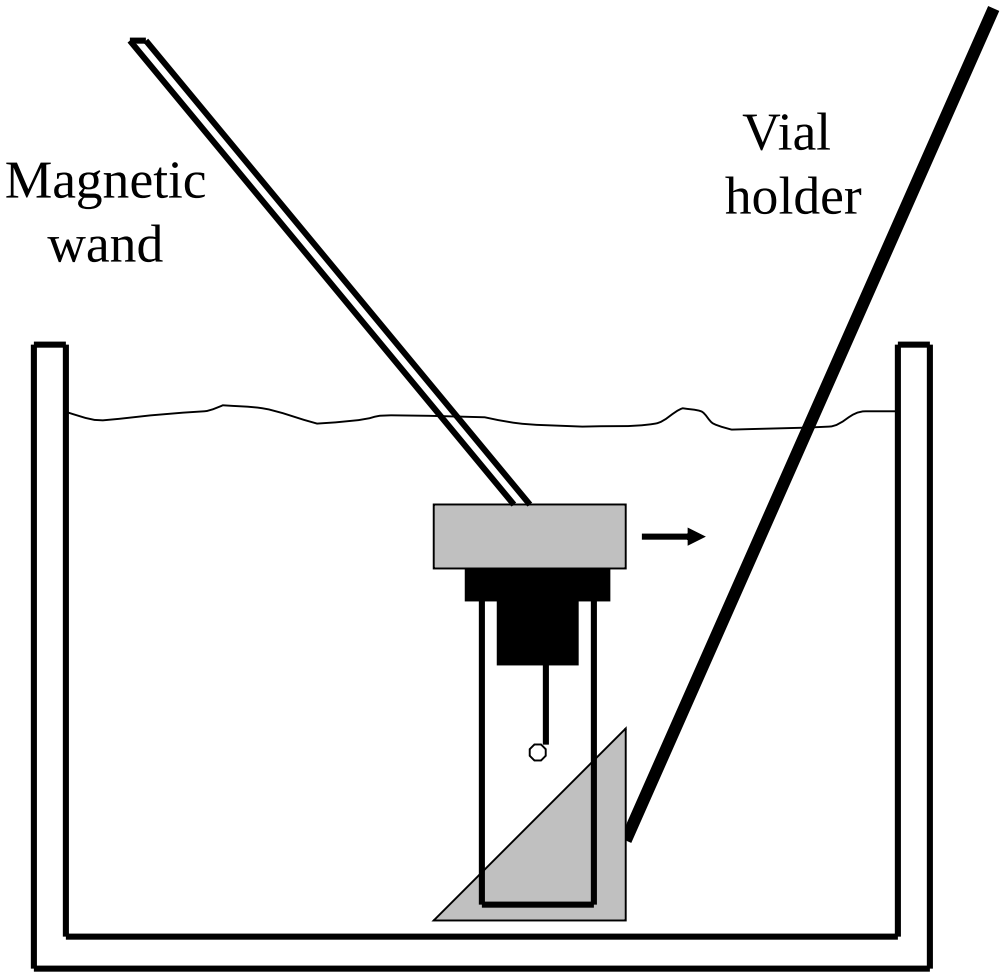
GOOGLE search (first hit):

ssrl dewar testing

[See also Owen, Pritchard & Garman. *J. Appl.Cryst.* (2004) **37**, 1000-1003]

Change storage LN_2 every 3 months.

c)



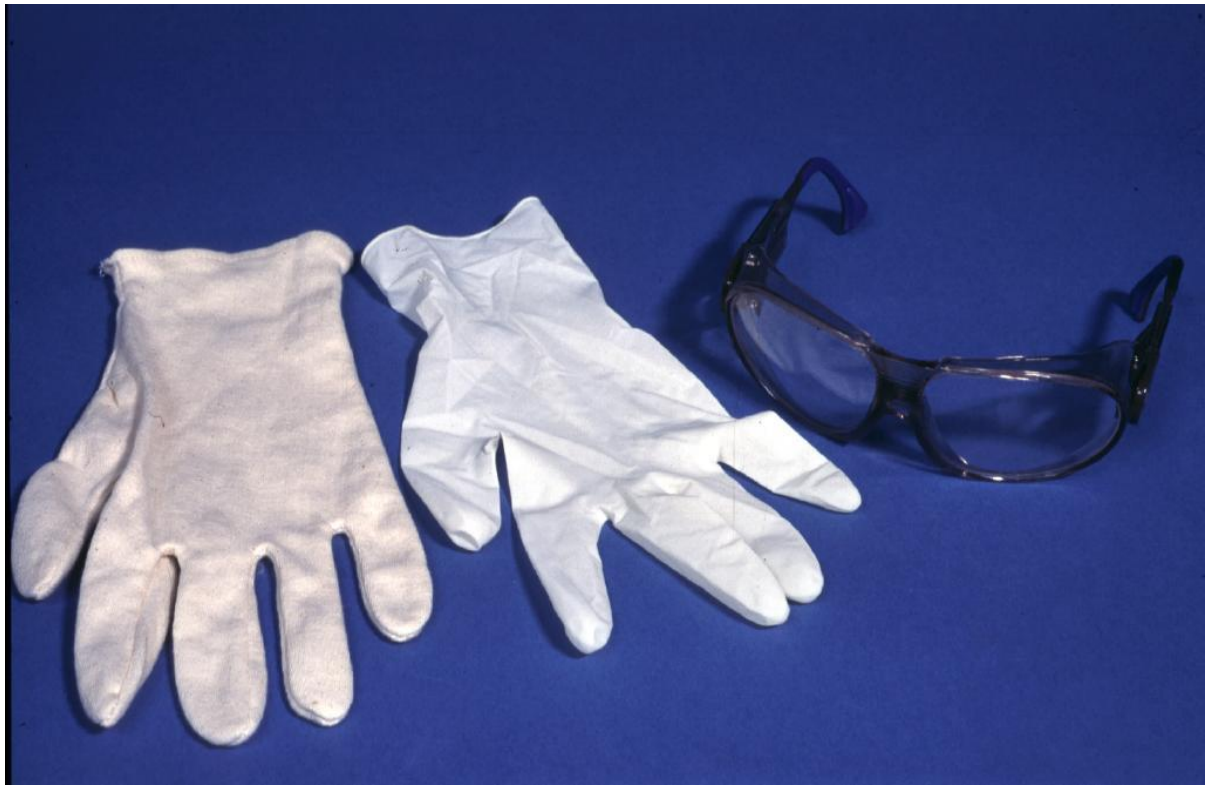
Crystal Handling under LN2

- Use appropriate tools
- Have a small working Dewar: change the LN2 in it often.
- Wait for LN2 to stop bubbling before trying any manipulations.
- Only move one hand at a time
- Steady the stationary hand on edge of Dewar

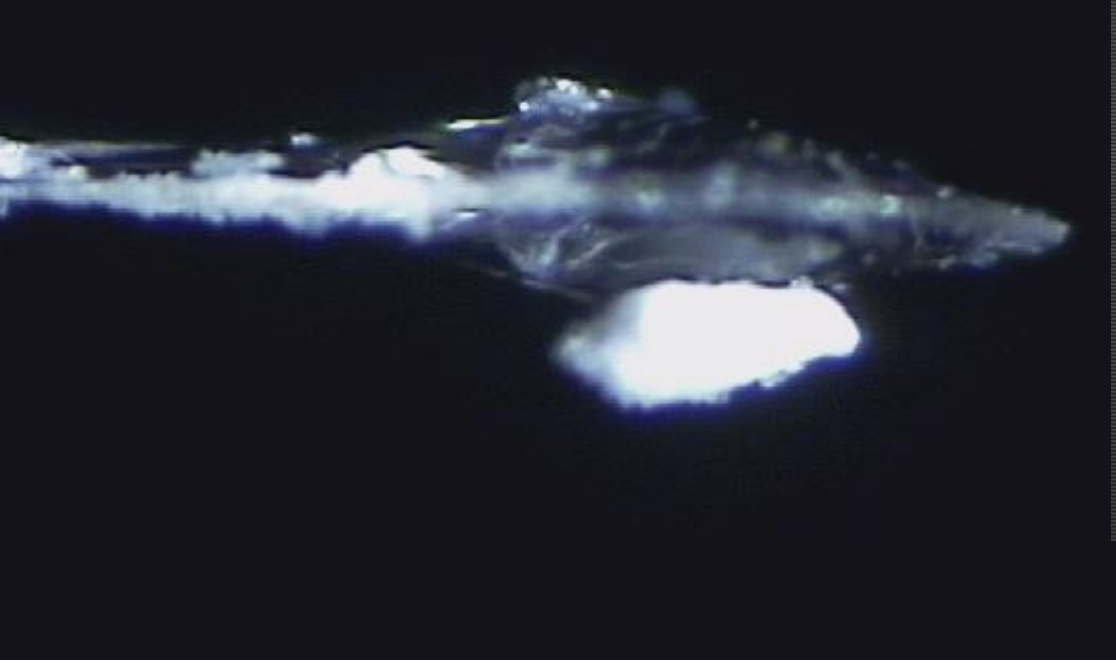
Step by step instructions:

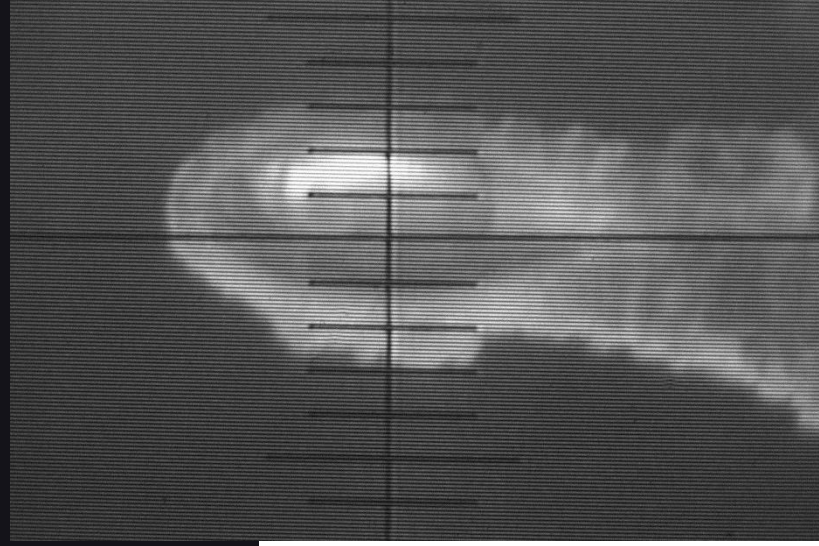
http://www.oxcryo.com/about/lmb-guide/

SAFETY: gloves and goggles

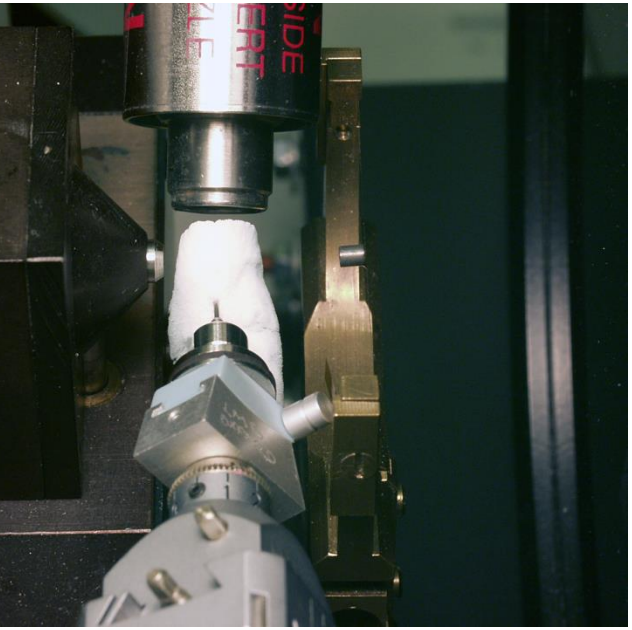


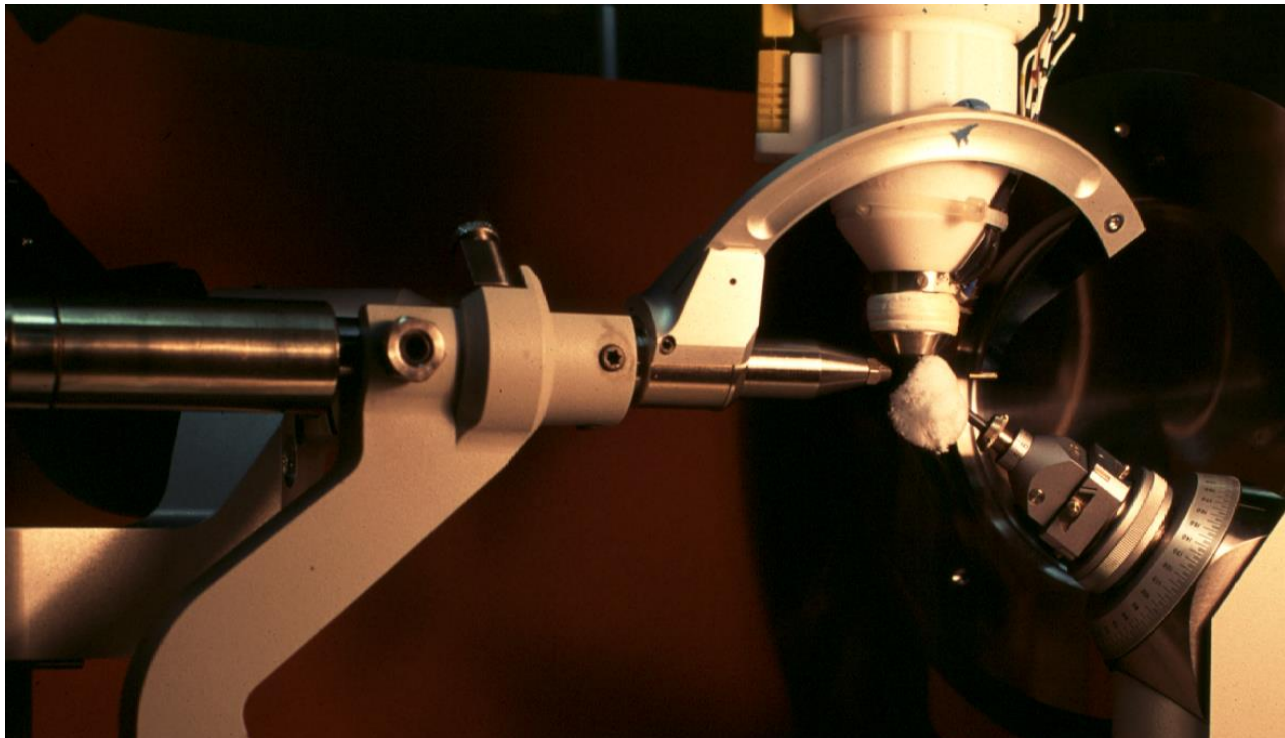
What am I doing wrong?





Absolutely NO ice of ANY sort.





CRYSTAL SOAKS:

- Use same cooling protocol
  [soak time, solution concentrations]
  BEST to use same human being too!

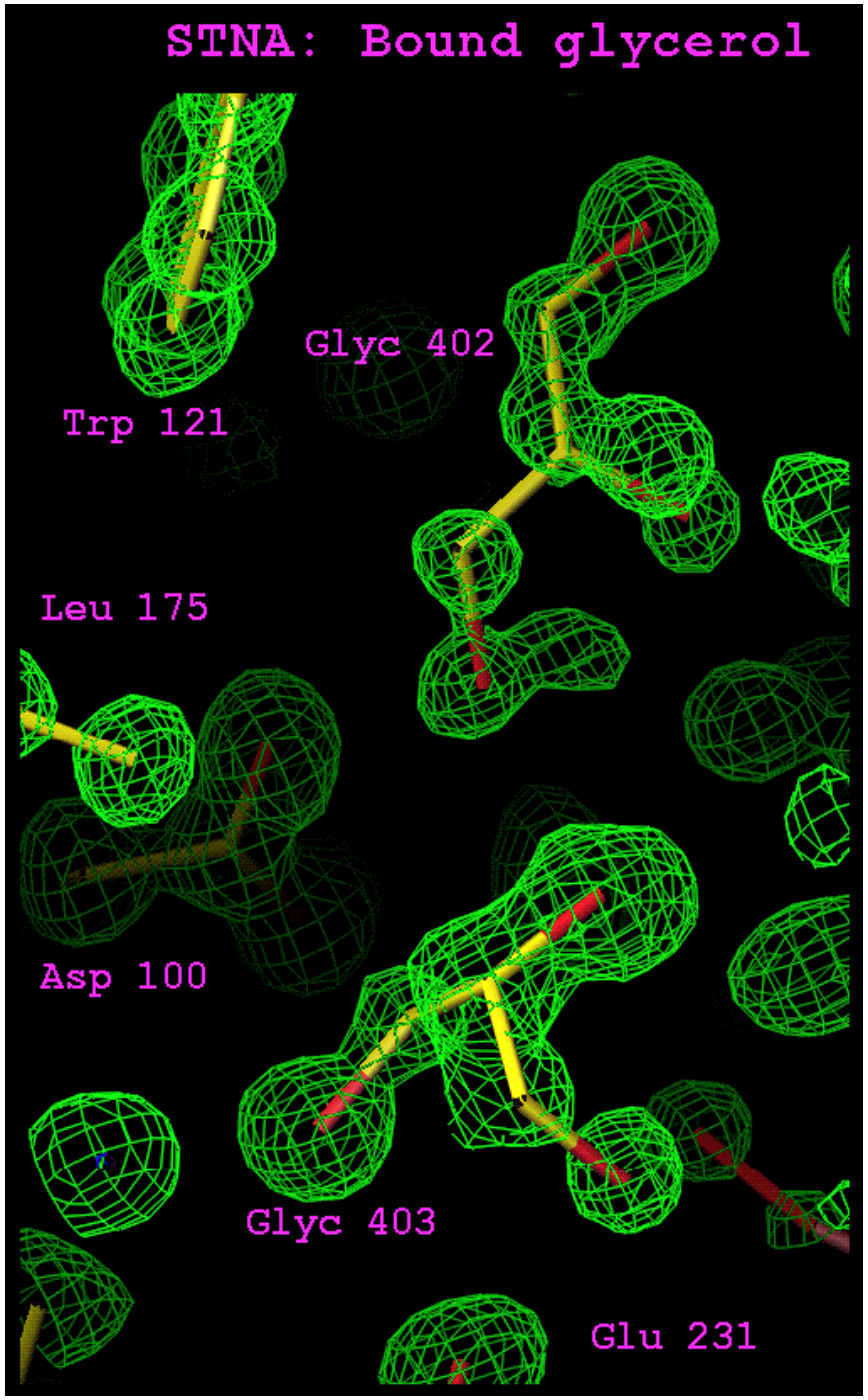
- MIR/substrate- put it in the cryo-buffer too:
  N.B.competitive inhibition with cryo-agent.

- Non-isomorphism: MR to native first

[Crick and Magdoff 1956]

Bound cryoprotectant agent.

Beware competitive inhibition with wanted substrate. Put substrate in cryo-buffer.



The Plan:

- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - Testing at room temperature.
  - Storage and retrieval.
  - **If nothing seems to work….**

If nothing seems to work:

- **Diffraction at room temperature?**
- **Cryo-solutions**
- **Transfer/handling/soaking – vary time and temperature [e.g. try 4º overnight]**
- **Cryogen choice: nitrogen gas/liquid**
- Osmolarity matching
- Crystal annealing
- Swap buffer
- Try more than once.

Theoretical study: starts to rationalise experimental practice.

Heat transfer study by a proper mechanical engineer!

Most to least important factors:

1) Crystal solvent content and solvent composition.
2) Crystal size and shape.
3) Amount of residual liquid around the crystal
4) Cooling method (liquid plunge *versus* gas stream).
5) Choice of gas/liquid.
6) Relative speed between cooling fluid and crystal.

[S. Kriminski, M. Kazmierczak and R.E. Thorne

`Heat transfer from protein crystals: implications for flash-cooling and X-ray beam heating. Acta Cryst. (2003) **D59**, 697-708.]

If nothing seems to work:

- Diffraction at room temperature?
- Cryo-solutions
- Transfer/handling/soaking – vary time and temperature [try 4º overnight]
- Cryogen choice
- **Osmolarity matching**
- **Crystal annealing**
- Swap buffer
- Try more than once.

Osmolarity matching:

1) Look up osmotic pressure of mother liquor in CRC Handbook of Physics and Chemistry, Section D-232. 11th column is O: Os/kg

2) Look up o.p. of cryoprotectant agent.

3) Modify conc. of mother liquor to minimise change in osmotic pressure.

Osmotic shock: compresses crystal $\Rightarrow$ cracks

$\Rightarrow$Mosaic spread increases $\Rightarrow$ resolution lower.

`Quick dips' give greatest osmotic shock but minimise time for cryoprotectant attack.

23 GLYCEROL, $CH_2OHCHOHCH_2OH$

MOLECULAR WEIGHT = 92.09
RELATIVE SPECIFIC REFRACTIVITY = 1.109

0.00 % by wt. d
For Values of 0

A % by wt.	ρ D_4^{20}	D_{20}^{20}	C_v g/l	M g-mol/l	C_w g/l	$(C_v - C_w)$ g/l	$(n - n_0) \times 10^4$	n	Δ °C	O Osm/kg	S g-mol/l	η/η_0
0.50	0.9994	1.0011	5.0	0.054	994.4	3.9	6	1.3336	0.072	0.039	0.020	1.009
1.00	1.0005	1.0023	10.0	0.109	990.5	7.7	12	1.3342	0.180	0.097	0.051	1.020
2.00	1.0028	1.0046	20.1	0.218	982.7	15.5	23	1.3353	0.411	0.221	0.119	1.046
3.00	1.0051	1.0069	30.2	0.327	974.9	23.3	35	1.3365	0.627	0.337	0.182	1.072
4.00	1.0074	1.0092	40.3	0.438	967.1	31.2	46	1.3376	0.849	0.456	0.247	1.098
5.00	1.0097	1.0115	50.5	0.548	959.2	39.0	58	1.3388	1.078	0.580	0.315	1.125
6.00	1.0120	1.0138	60.7	0.659	951.3	46.9	70	1.3400	1.316	0.708	0.385	1.155
7.00	1.0144	1.0162	71.0	0.771	943.4	54.9	82	1.3412	1.561	0.839	0.457	1.186
8.00	1.0167	1.0185	81.3	0.883	935.4	62.9	94	1.3424	1.811	0.974	0.530	1.218
9.00	1.0191	1.0209	91.7	0.996	927.4	70.9	106	1.3436	2.064	1.110	0.603	1.253
10.00	1.0215	1.0233	102.1	1.109	919.3	78.9	118	1.3448	2.323	1.249	0.678	1.288
12.00	1.0262	1.0281	123.1	1.337	903.1	95.1	142	1.3472	2.880	1.548	0.837	1.362
14.00	1.0311	1.0329	144.4	1.568	886.7	111.5	167	1.3496	3.469	1.865	1.004	1.442
16.00	1.0360	1.0378	165.8	1.800	870.2	128.0	191	1.3521	4.094	2.201	1.177	1.530
18.00	1.0409	1.0428	187.4	2.035	853.6	144.7	217	1.3547	4.756	2.557	1.359	1.627
20.00	1.0459	1.0478	209.2	2.272	836.8	161.5	242	1.3572	5.46	2.93	1.546	1.734
24.00	1.0561	1.0580	253.5	2.752	802.6	195.6	294	1.3624	7.01	3.77	1.944	1.984
28.00	1.0664	1.0683	298.6	3.243	767.8	230.4	347	1.3676	8.77	4.71	2.370	2.274
32.00	1.0770	1.0789	344.6	3.742	732.3	265.9	400	1.3730	10.74	5.78	2.814	2.632
36.00	1.0876	1.0896	391.5	4.252	696.1	302.2	455	1.3785	12.96	6.97	3.276	3.082
40.00	1.0984	1.1003	439.4	4.771	659.0	339.2	511	1.3841	15.50	8.33	3.757	3.646
44.00	1.1092	1.1112	488.1	5.300	621.2	377.1	567	1.3897				4.434
48.00	1.1200	1.1220	537.6	5.838	582.4	415.8	624	1.3954				5.402
52.00	1.1308	1.1328	588.0	6.385	542.8	455.4	681	1.4011				6.653
56.00	1.1419	1.1439	639.4	6.944	502.4	495.8	739	1.4069				8.332
60.00	1.1530	1.1551	691.8	7.513	461.2	537.0	799	1.4129				10.66
64.00	1.1643	1.1663	745.1	8.091	419.1	579.1	859	1.4189				13.63
68.00	1.1755	1.1775	799.3	8.680	376.1	622.1	919	1.4249				18.42
72.00	1.1866	1.1887	854.3	9.277	332.2	666.0	980	1.4310				27.57
76.00	1.1976	1.1997	910.2	9.883	287.4	710.8	1040	1.4370				40.49
80.00	1.2085	1.2106	966.8	10.498	241.7	756.5	1101	1.4431				59.78
84.00	1.2192	1.2214	1024.2	11.121	195.1	803.2	1162	1.4492				84.17
88.00	1.2299	1.2320	1082.3	11.752	147.6	850.7	1223	1.4553				147.2
92.00	1.2404	1.2426	1141.1	12.392	99.2	899.0	1284	1.4613				383.7
96.00	1.2508	1.2530	1200.7	13.039	50.0	948.2	1344	1.4674				778.9
100.00	1.2611	1.2633	1261.1	13.694	0.0	998.2	1405	1.4735				1487.0

24 HYDROCHLORIC ACID, HCl

Osmotic Pressure Matching: Example

Osmality: Os/kg

- 2.0M NaCl

  50mM pH 7.8 Tris HCl 3.95

- Need 20% glycerol 2.9

  1.05

From Tables, 0.55M NaCl has o.p. 1.05 Os/kg

$\Rightarrow$Try 0.55 M NaCl, 20% glycerol

50mM pH 7.8 Tris HCl

Crystal annealing: remove ice, reduce mosaicity and increase resolution

- Block stream temporarily (1-10 secs) OR
- Put crystal back in cryo-buffer solution.
- Then flash cool again.
- Worth a try (can repeat several times).
- Works sometimes.

  [misnomer: slow heat and fast cool is really `tempering']

Yeh and Hol (1998) *Acta Cryst*. D54, 479- 480.

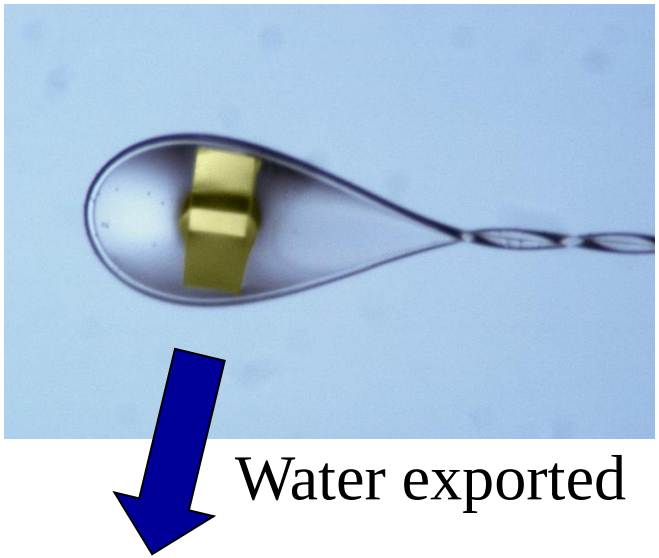
Harp *et al*. (1998) *Acta Cryst* D54, 622-628, and (1999) D55, 1129-1134.

Hanson *et al*. (2003) *Meth Enzym* 368, 217

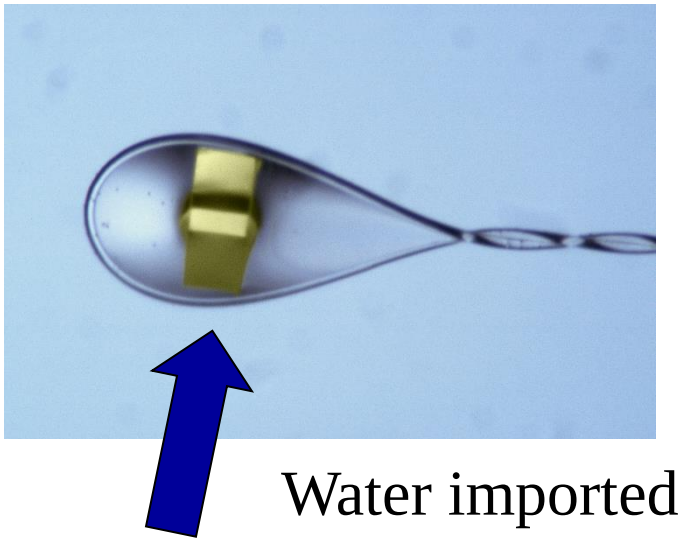
Understanding why annealing sometimes works.

Cryoprotectant agent concentration: match contraction of lattice to contraction of bulk solvent to avoid lattice distortion, higher mosaicity etc.

Not enough cryoprotectant



Too much cryoprotectant

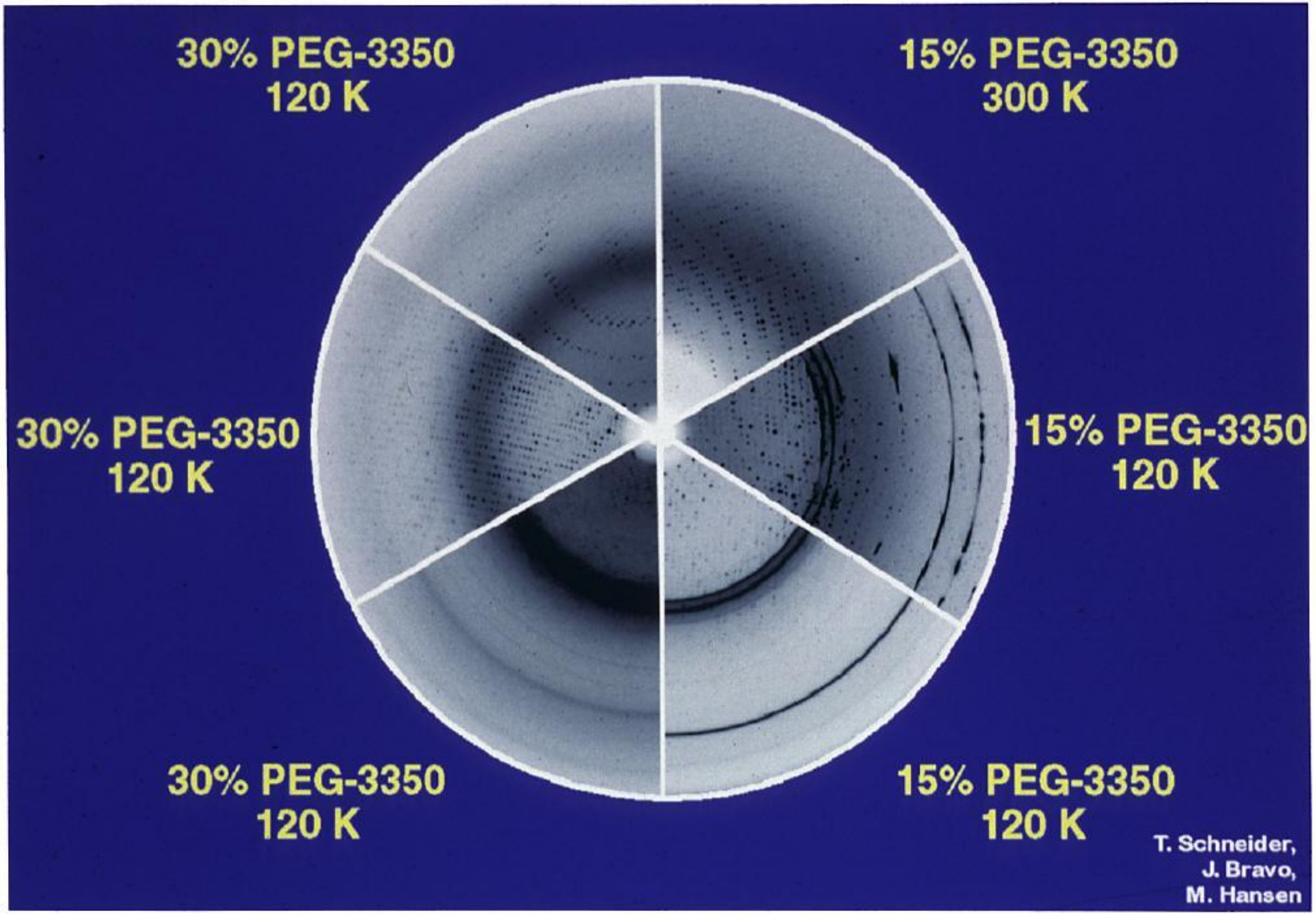


Juers and Matthews, Acta Cryst (2004) 412-421.

If nothing seems to work:

- Diffraction at room temperature?
- Cryo-solutions
- Transfer/handling/soaking – vary time and temperature [4º]
- Cryogen choice
- Osmolarity matching
- Crystal annealing
- **Swap buffer**
- **Try more than once**.

Have at least 6 goes…



CRYO-COOLING:

Advantages

- Reduced radiation damage.
- Gentler mounting
- Lower background
- Higher resolution
- Fewer crystals
- Can ship crystals
- Use crystals when ready.

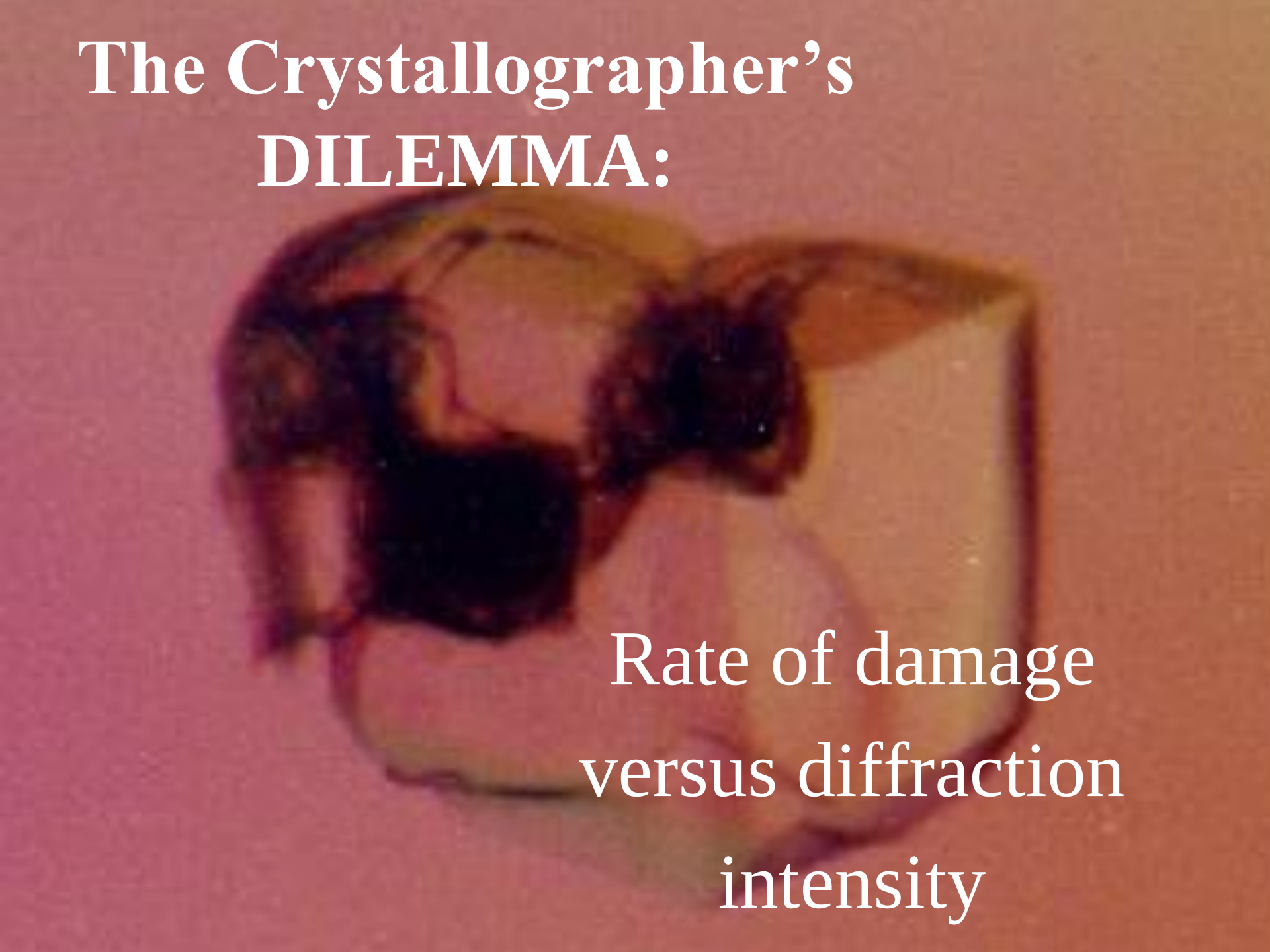
Disadvantages

- Expensive equipment
- Increase in mosaic spread.
- Need to invest time.

Cryocrystallography: for more gory details see:

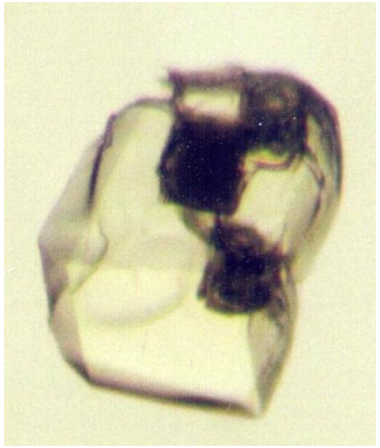
- Rodgers, D (1997) Methods in Enzymology 276, 183-203 and in International Tables of Crystallography Volume F (2001) 202-208.
- Hope, H in International Tables of Crystallography Volume F (2001) 197-201.
- Garman and Schneider (1997) J.Appl. Cryst, 30, 211-237.
- Parkin and Hope (1998) J.Appl. Cryst. 31, 945-953.
- Garman (1999) Acta D55, 1641-1653.
- Garman and Doublié (2003) Methods in Enzym. 396, 188-216.
- Garman (2003) Curr. Opin. Struct. Biol. 13, 545-551.
- Garman and Owen, (2006) *Acta Cryst. D* 62, 32-47.
- Pflugrath (2015) *Acta Cryst F* 71, 622-642.

The Crystallographer's DILEMMA:



Rate of damage versus diffraction intensity

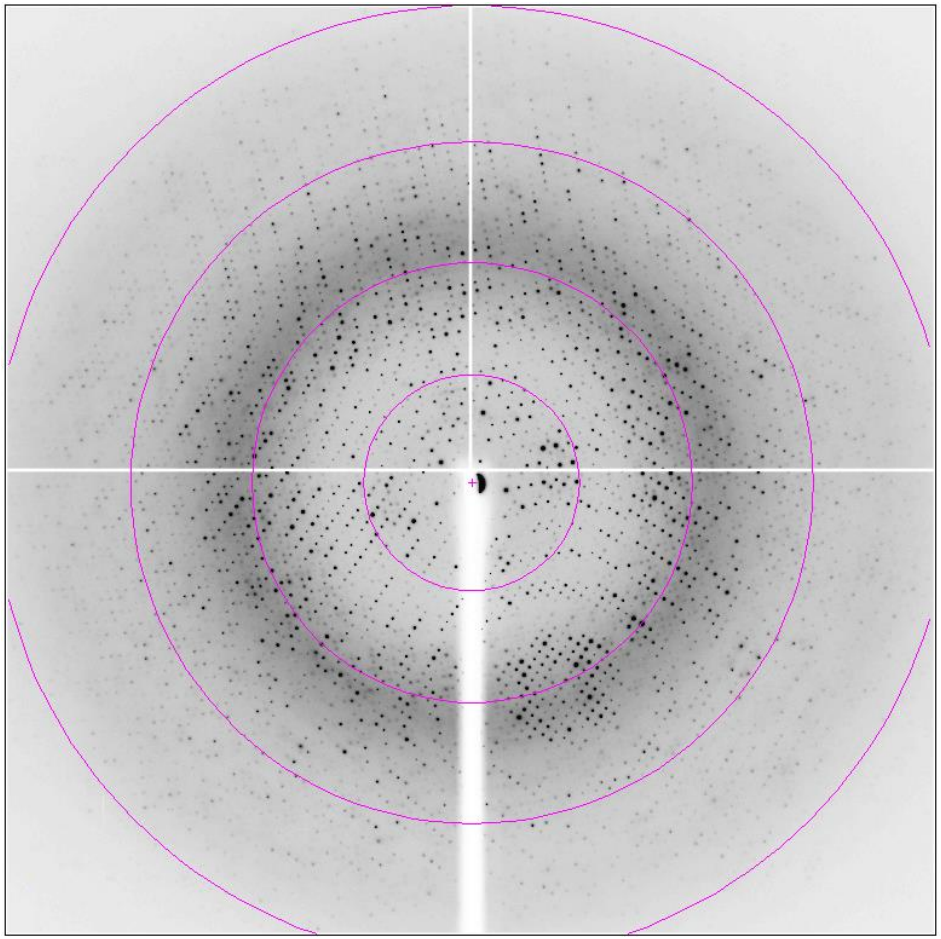
Still the first day and my brain is already over full…



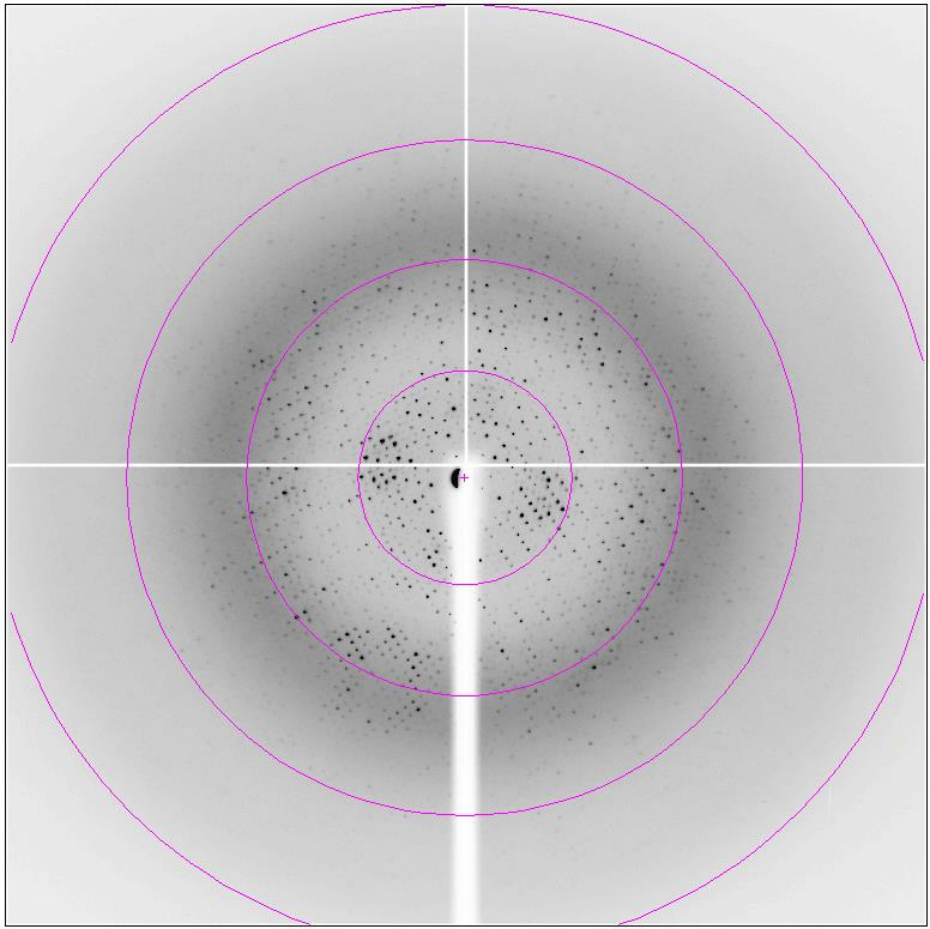
Radiation damage:

The Plan:

- **What are the symptoms?**
- What is it?
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?



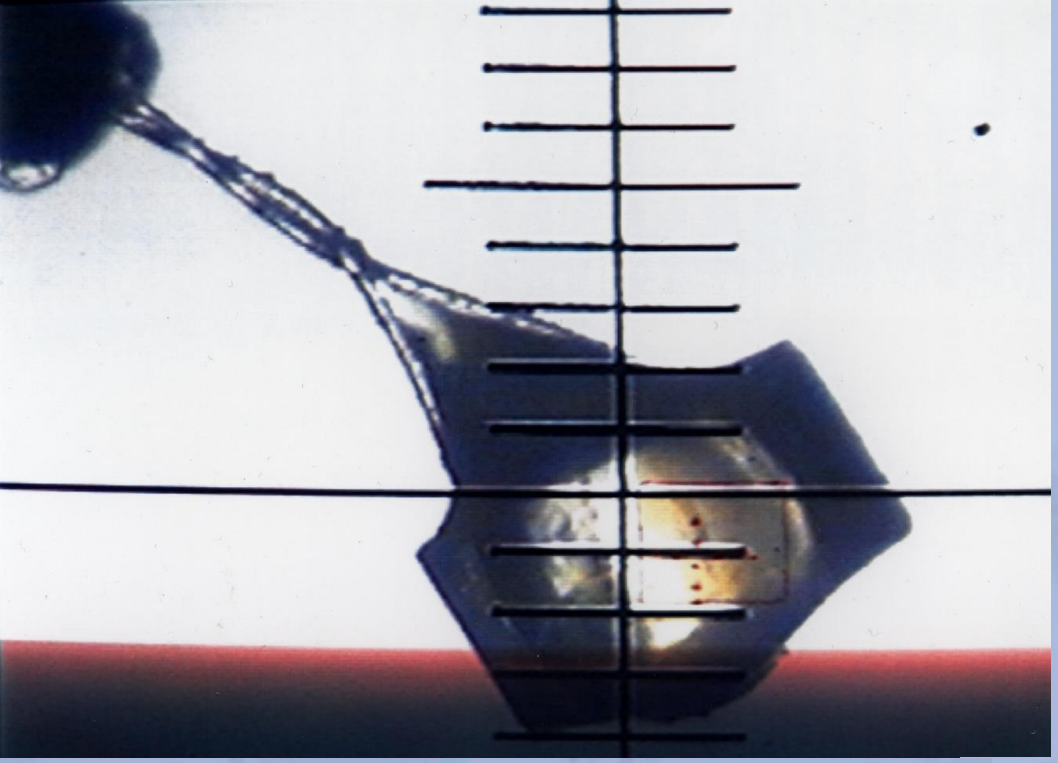
Intensity
decrease



RT

Loss of
diffraction

Incomplete data
from crystals

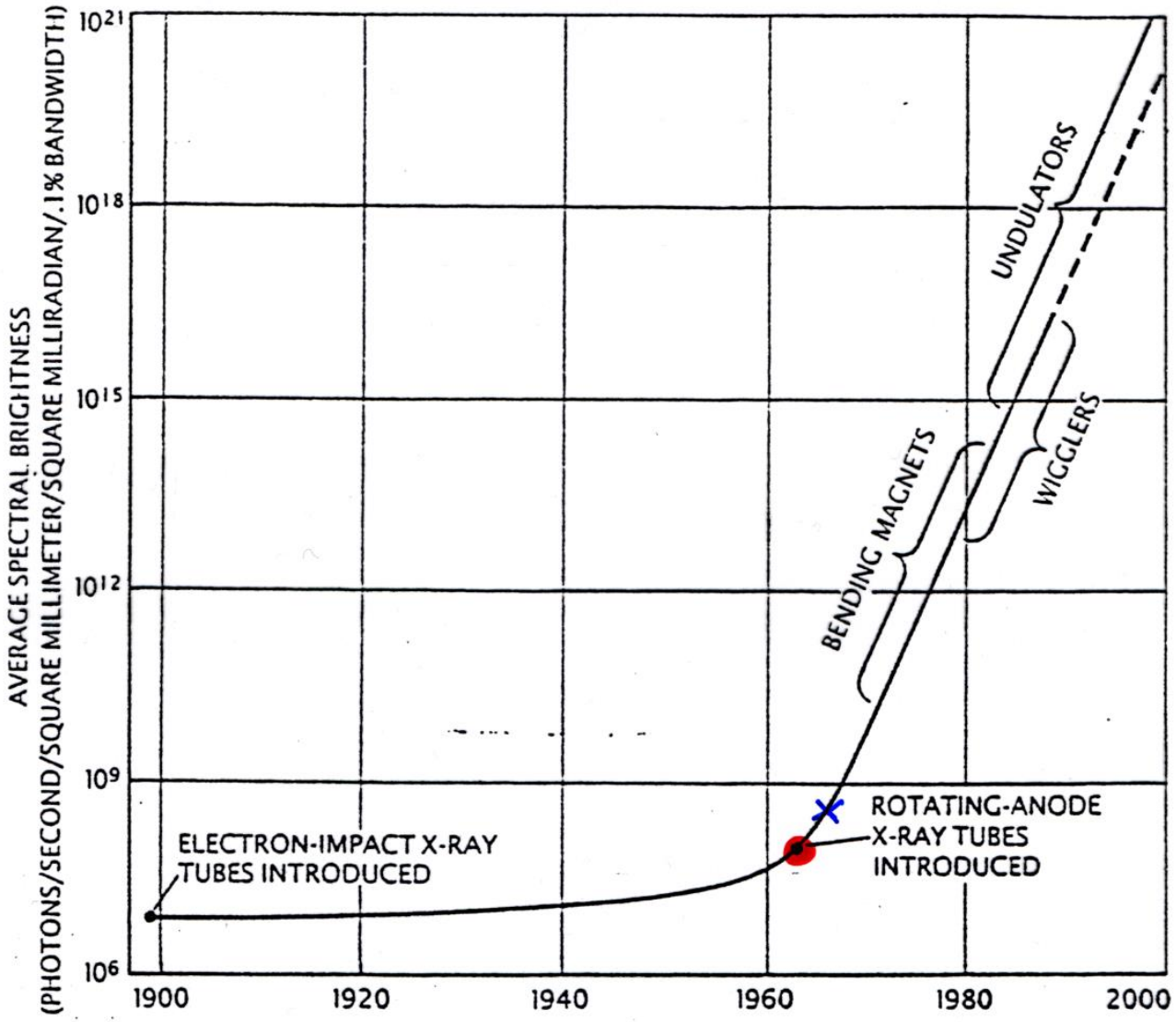


A

B

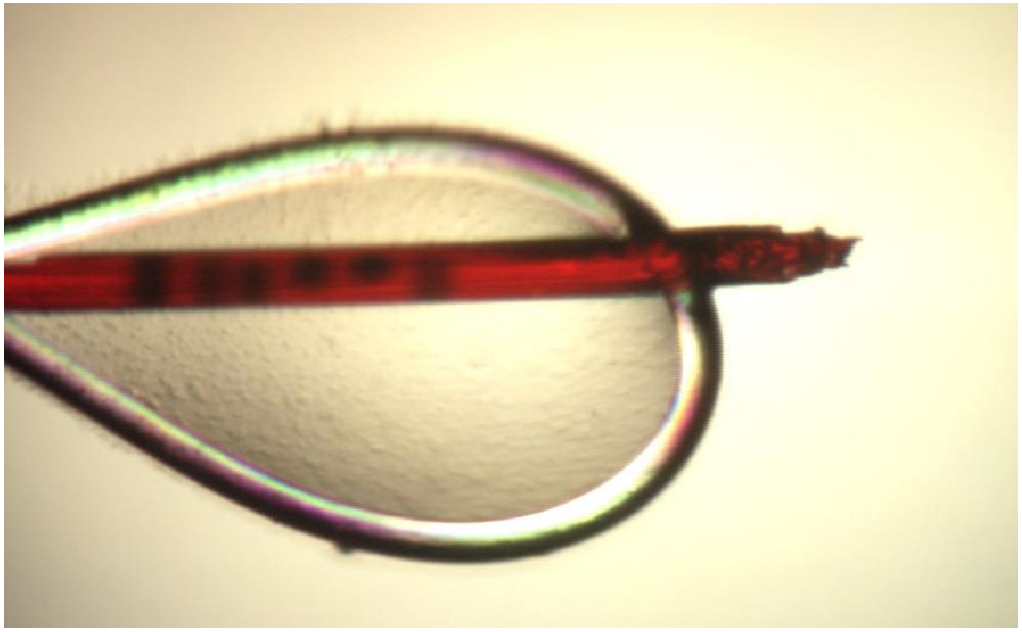
C

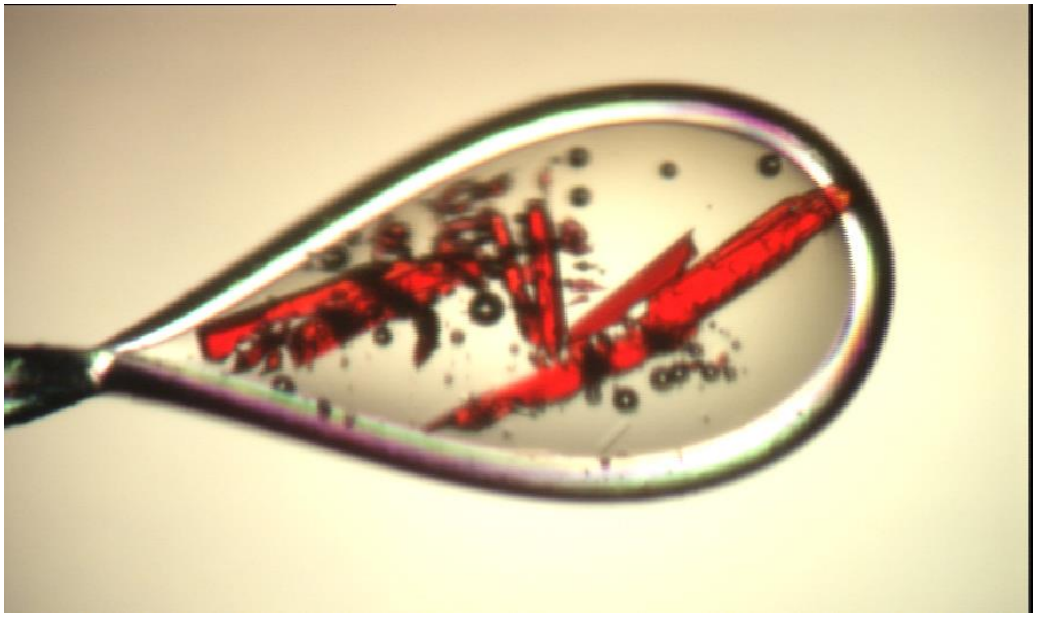
SSRL, 19 hours, 9.1, 1998



BRIGHTNESS OF X RAYS has increased by many orders of magnitude since the advent of synchrotron-radiation sources. Undulators in storage rings are the brightest source.

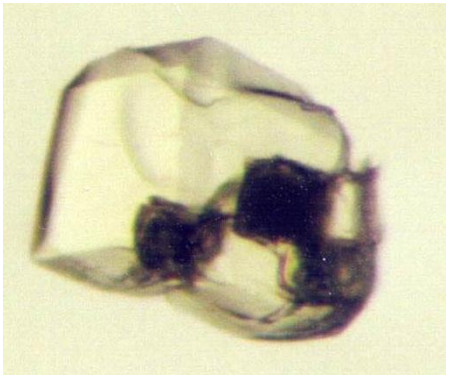
× multi-layer optics.





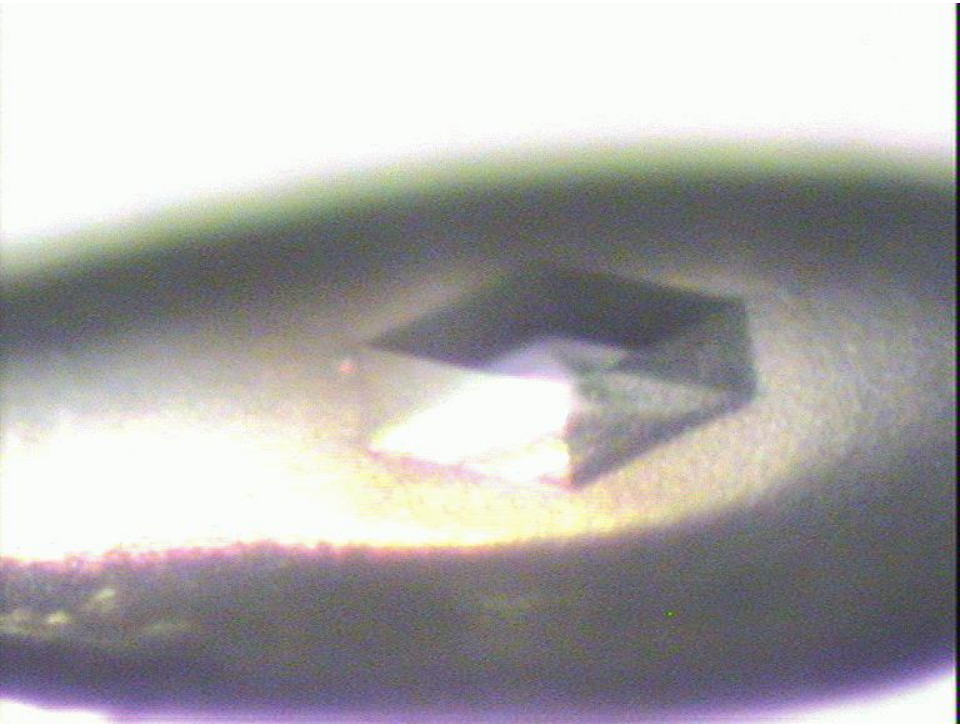
1995 onwards:
100 K

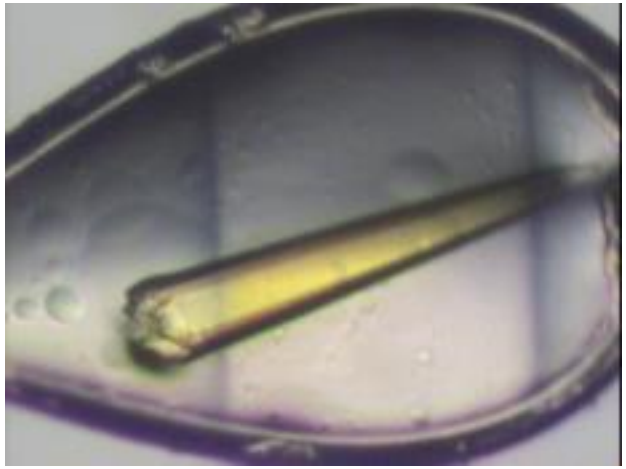
BUT THEN, 1999:



2002:
30μm beam

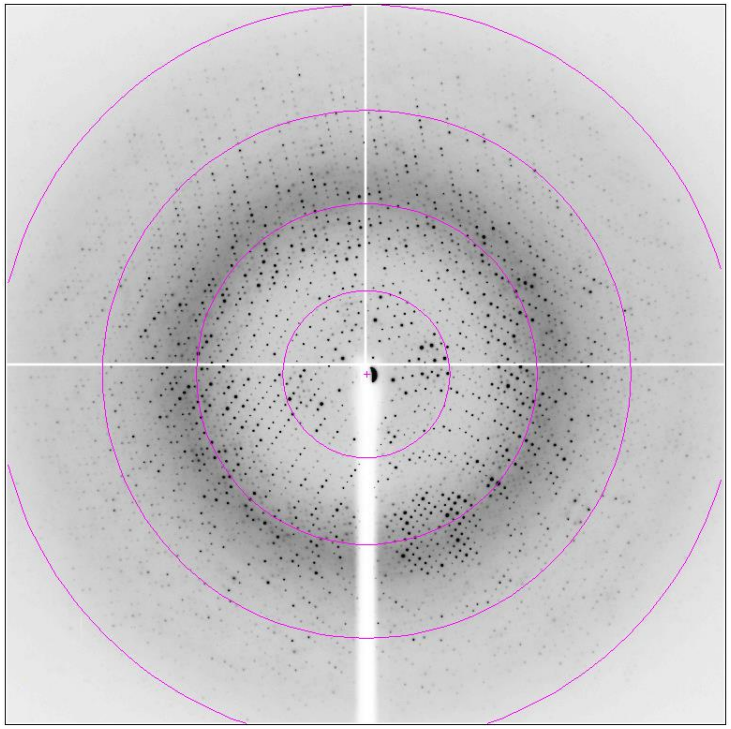
Iron containing protein, ESRF [Tassos Perrakis]





Also observe spectral changes

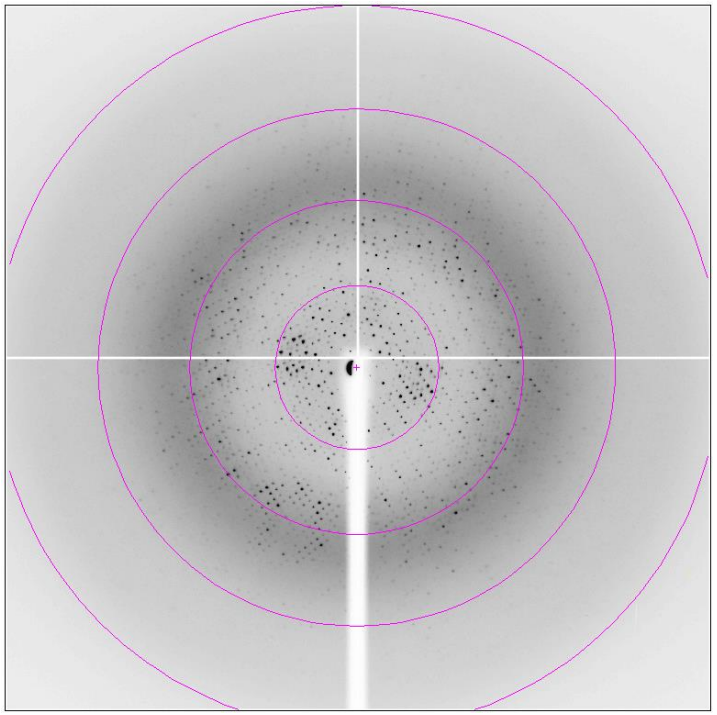
Garman and Owen (2006), Acta D62, 32-47.



Intensity decrease

Loss of diffraction

Incomplete data from crystals



Dataset 1

Dataset 10

Happens during 1 dataset at 100K for some crystals

Unit cell volume expansion

Increase in Wilson B factors

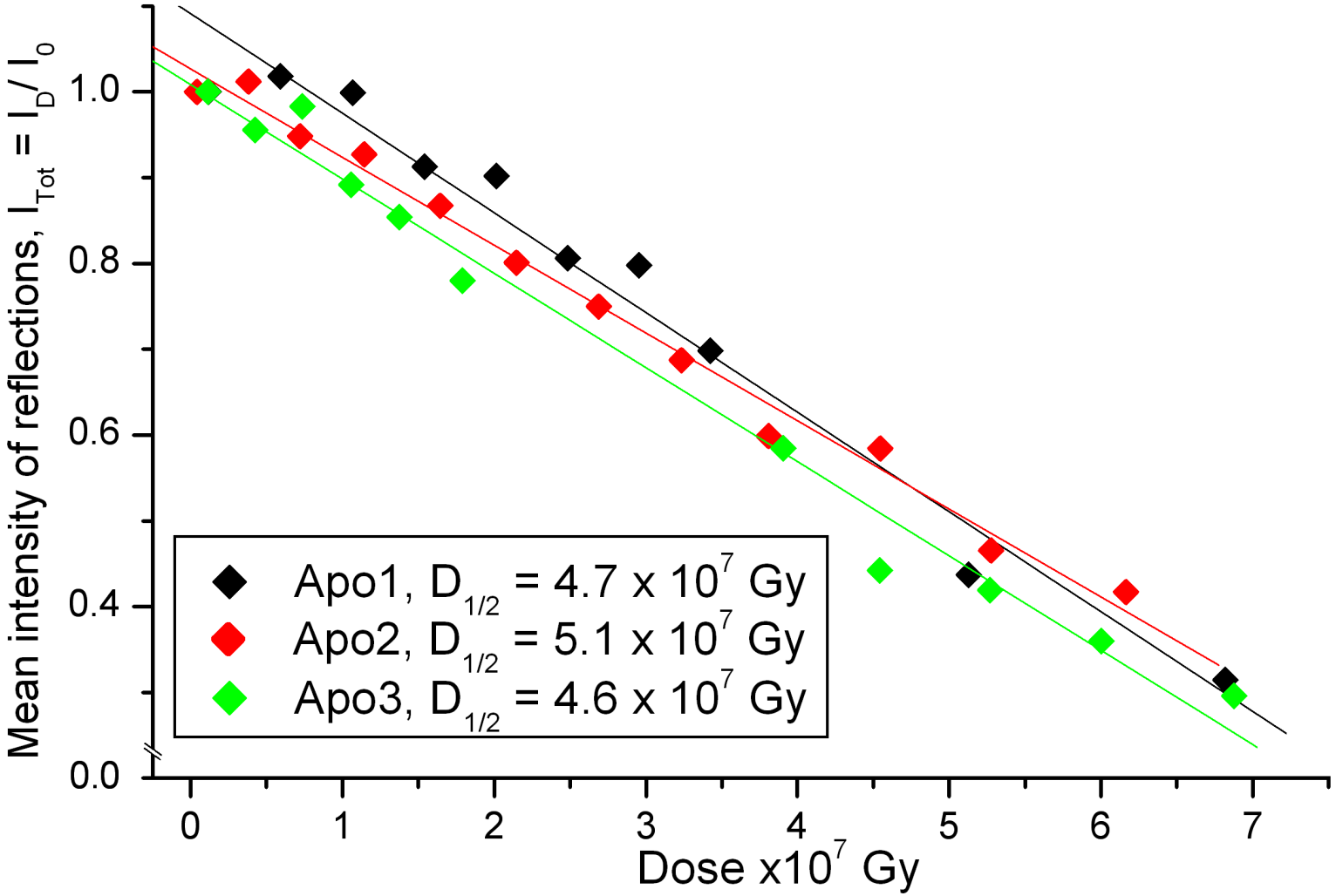
1×10^{12} ph s^{-1} into 100μm square slits

‘GLOBAL’ damage

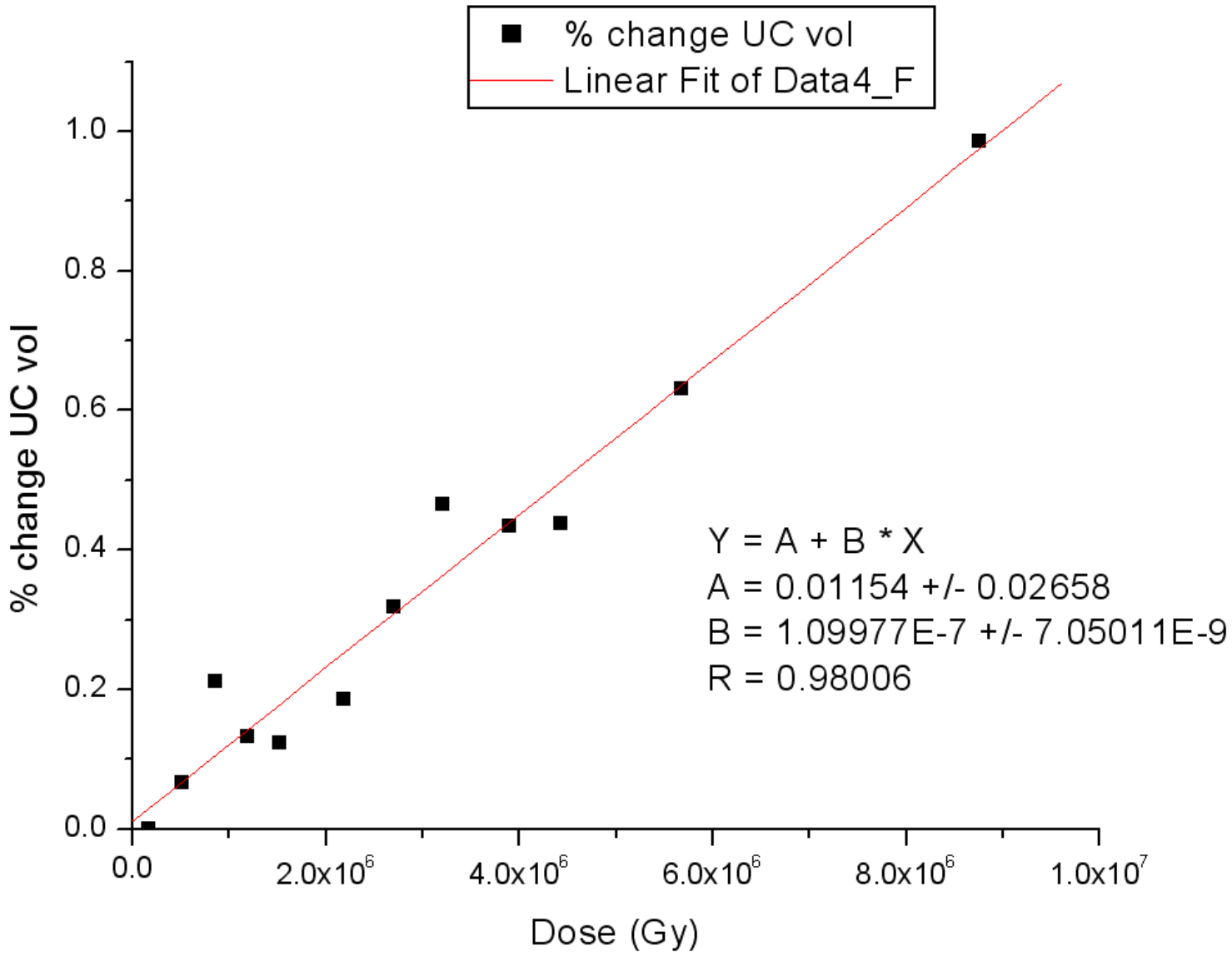
3×10^{13} ph s^{-1} into 50μm × 70μm [10^{14}]

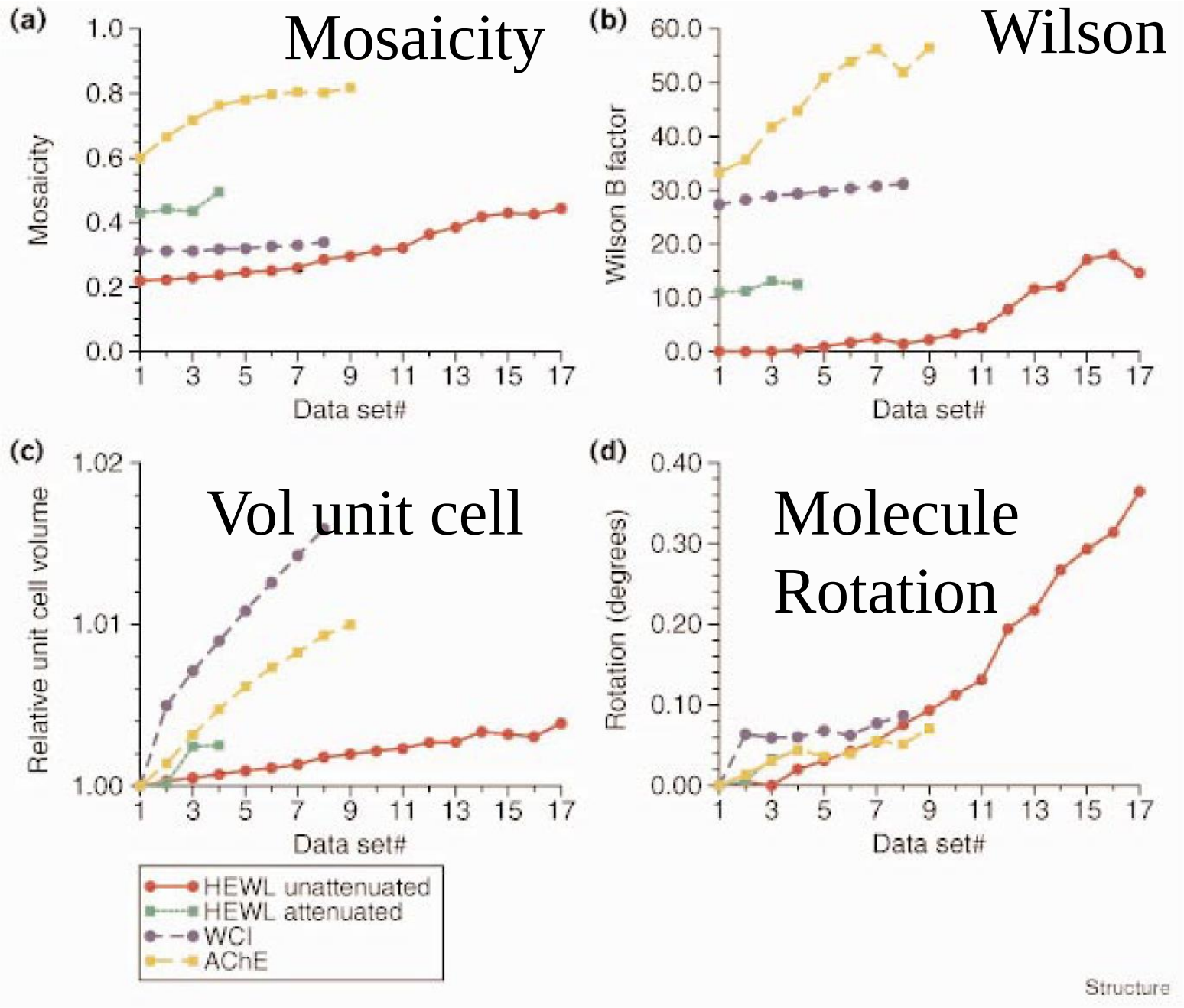
Intensity Decay at 100K

Normalised Intensity vs Dose: apoferritin



Unit cell volume increase





Ravelli and McSweeney, Structure (2000) 8, 315

Data Parameters affected by Radiation Damage

- I /σ(I) or resolution limit ↓
- R_{merge} ↑
- Scaling B factors ↑
- Mosaicity ↑
- Unit Cell expansion a) function of dose ↑

  b) function of cryogen temperature ↑

Could this be an on-line damage metric?

[Ravelli and McSweeney, (2000) Structure]

No!

[Murray and Garman (2002), JSR, Ravelli et al (2002) JSR]

What global damage metric should we use and against what should we plot it?

- I_n/I_o
- Not I/sigma(I)
- B factors?
- An R_{meas} type measure?

Dose estimation (the *x*-axis!)

$$\text{Dose} = \frac{\text{energy absorbed}}{\text{unit mass}}$$

$$= \frac{\text{J}}{\text{kg}} = \text{Gy}$$

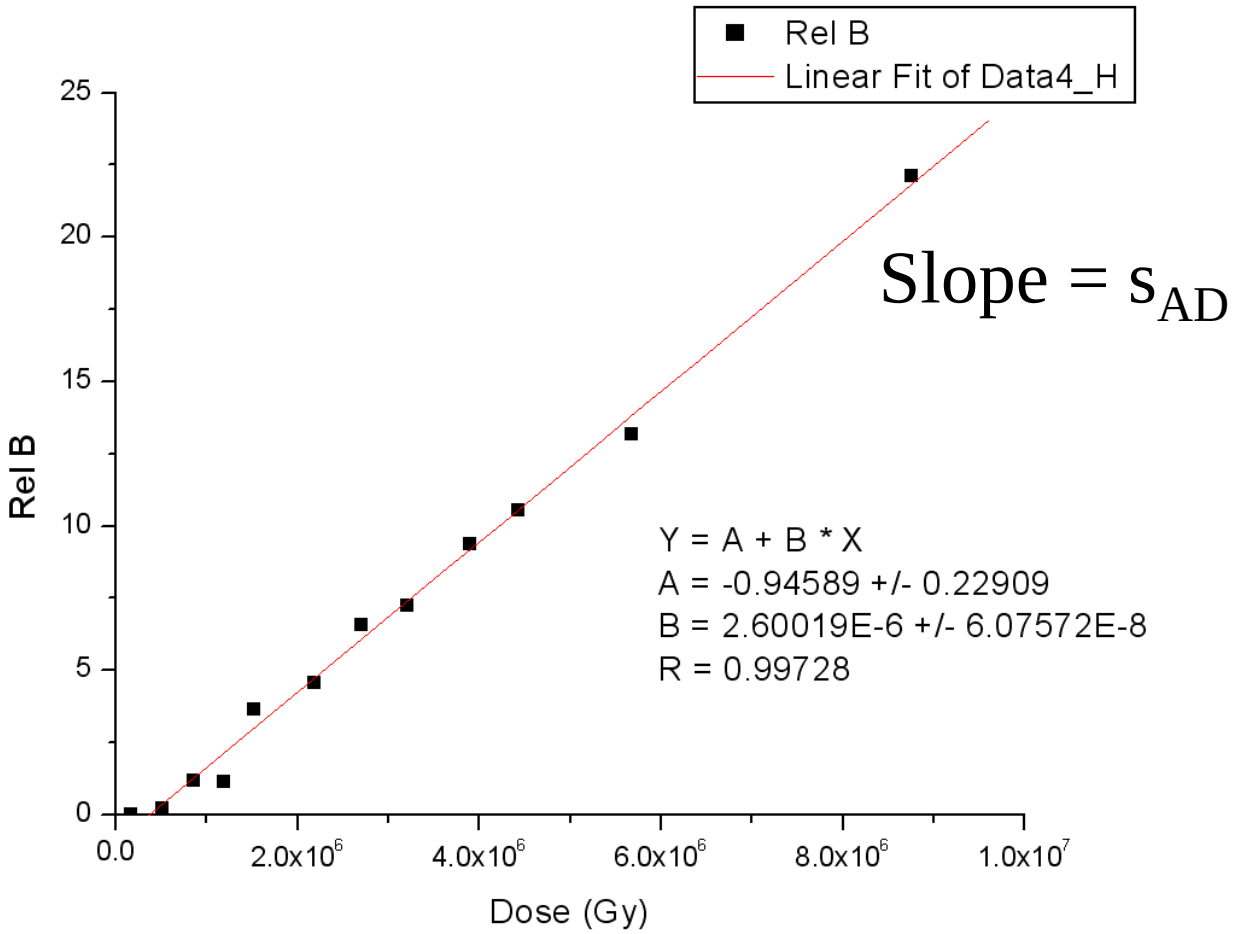
DOSE

- DOSE is the ENERGY lost per KILOGRAMME (!!)
- Measured in Joules/kg i.e. the absorbed energy per unit mass.
- Fundamental metric against which to measure damage.
- FLUX is in photons/second.
- Flux density is in photons/second/unit area.
- Dose takes care of the physics but NOT the chemistry.

Can define (to plot against dose):

- Coefficient of sensitivity α change in relative isotropic B factor: [Kmetko et al 2006, Acta D62, 1030]

$s_{AD} = \Delta B_{rel} / 8\pi^2 \Delta D$ (e.g. HEWL@100 K = 0.012 Å^2/Gy)

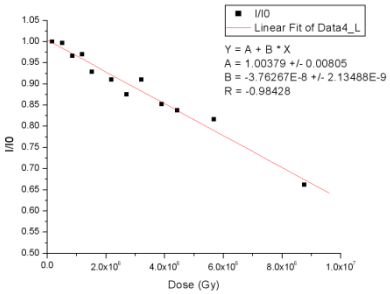


Global damage: summary

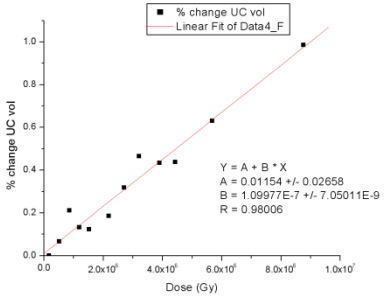
- Incomplete data.
- Causes non-isomorphism: MAD/SAD problems.
- No significant ($< \times 2$) dose rate effect at 100 K at current flux densities (10^{15} ph/s/mm^2).
- No significant ($< \times 2$) temperature dependence, but weak minimum at around 50 K.
- Damage to lattice due to hydrogen abstraction and then build up?
- Heating not significant at current flux densities.
- For a particular system is predictable/can be modelled (using a sacrificial crystal)

Changes upon X-ray exposure

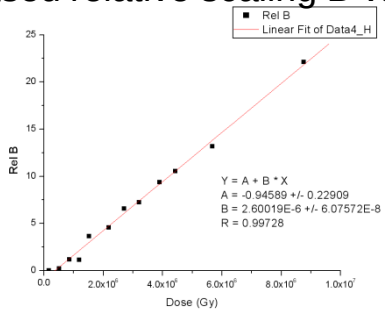
Relative intensity loss

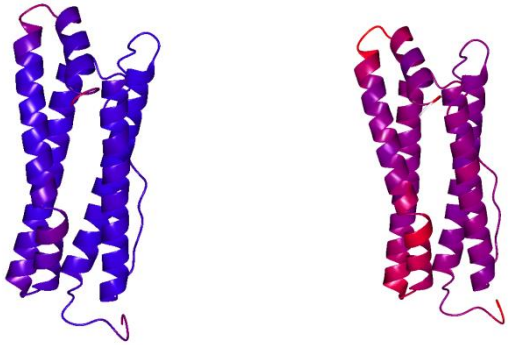


Increased unit cell volume



Increased relative scaling B values

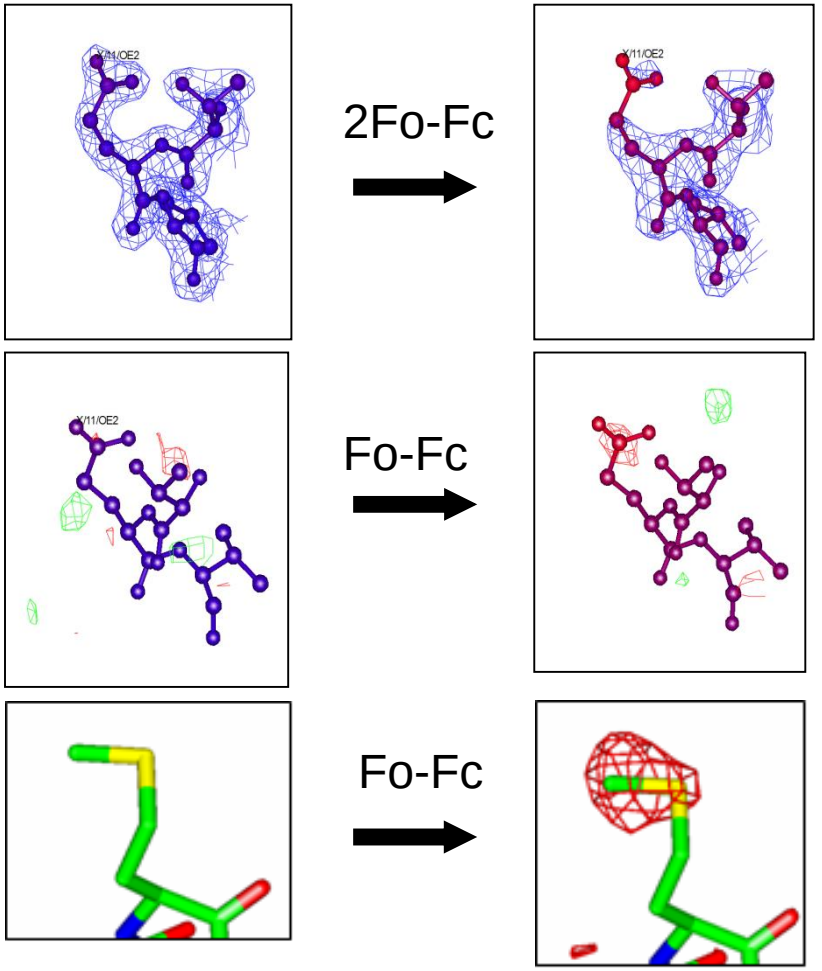




B values Å^2 (0=blue, 70 =red)

Ds1 Ds10

Electron density maps



- Loss of summed intensity
- Increased unit cell volume
- Increased B values
- Loss of electron density

Specific structural damage observed:

- Disulphide bridges broken
- Decarboxylation of glutamate and aspartate residues
- Tyrosine residues lose their hydroxyl group
- Methionines: carbon-sulphur bond cleaved

Weik *et al* (2000) PNAS 97, 623-628
Burmeister (2000), Acta Cryst D56, 328-341.
Ravelli and McSweeney, (2000) Structure 8, 315-328.

- Rupture of covalent bonds to heavier atoms:

C-Br, C-I, S-Hg

Note that if this were due to primary damage alone, damage would be in order of absorption cross sections of atoms, which it is not.

Specific Damage: summary.

- Can compromise biologically relevant observations (e.g. damage enzymatically important glutamates).
- Metallo-enzymes are reduced by X-ray beam.
- Perhaps weakly dose rate dependent ($< \times 2$)
- Perhaps weakly wavelength dependent ($< \times 2$)
- Weakly temperature dependent (varying results) ($< \times 2$)
- Can be reduced with certain scavengers: but very conflicting results (mainly $< \times 2$, benzoquinone RT $\times 9$)
- We DON'T understand pecking order of damage within an amino acid group pH? Solvent accessibility? Neighbouring amino acids?

Radiation damage:

The Plan:

- What are the symptoms?
- **What is it?**
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?

PHYSICS of the interaction of X-rays with crystals.

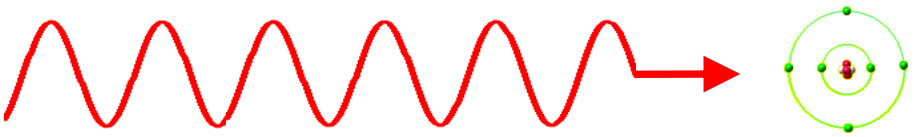
A) Diffraction

B) Absorption = Energy loss

N.B. > 90% of the beam does not interact at all, but goes straight through.

A) Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

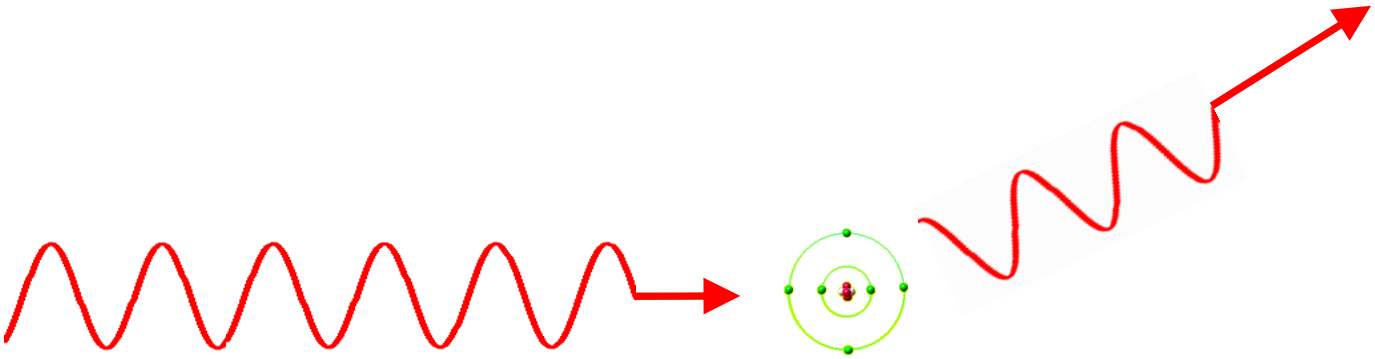


[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

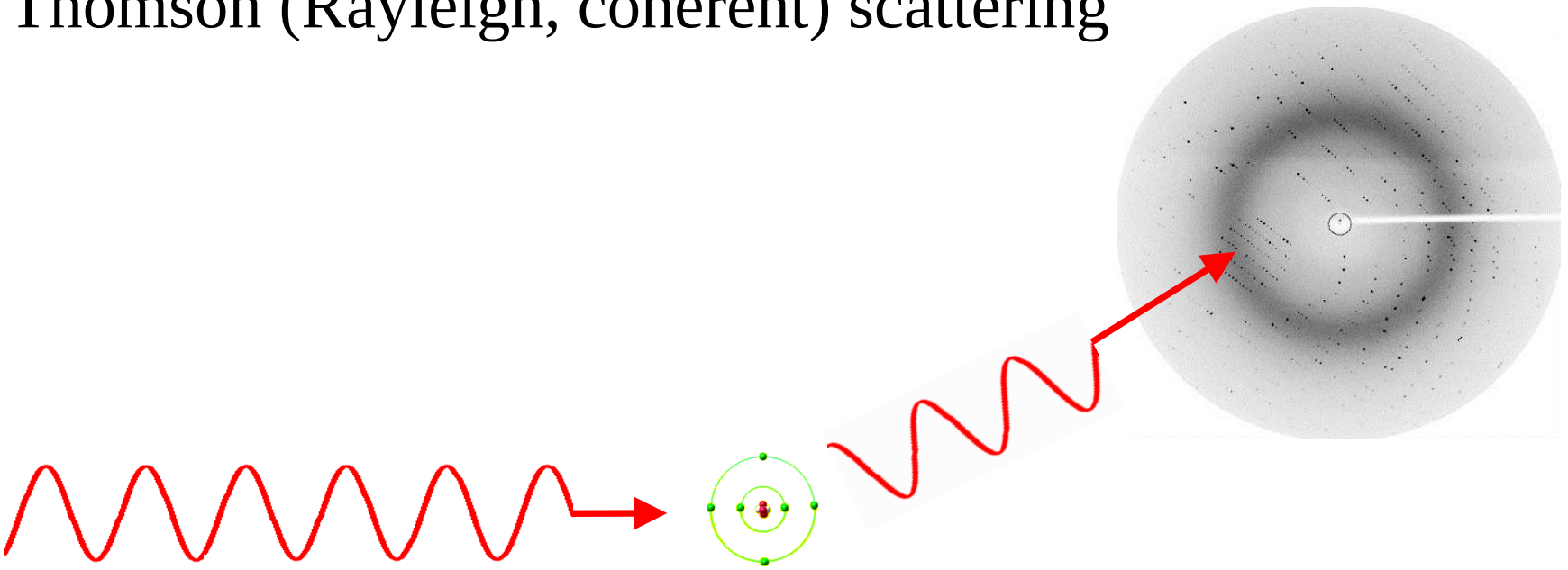


[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering



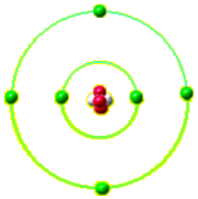
ELASTIC - no energy loss.

Coherent – adds vectorially and gives diffraction pattern.

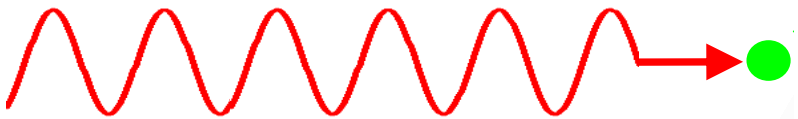
Small proportion of total scattering: 8% at 1Å

BUT IT IS THE BIT WE WANT!!

Compton (incoherent) scattering

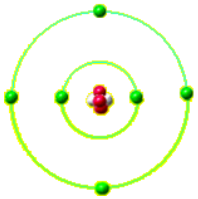


?!

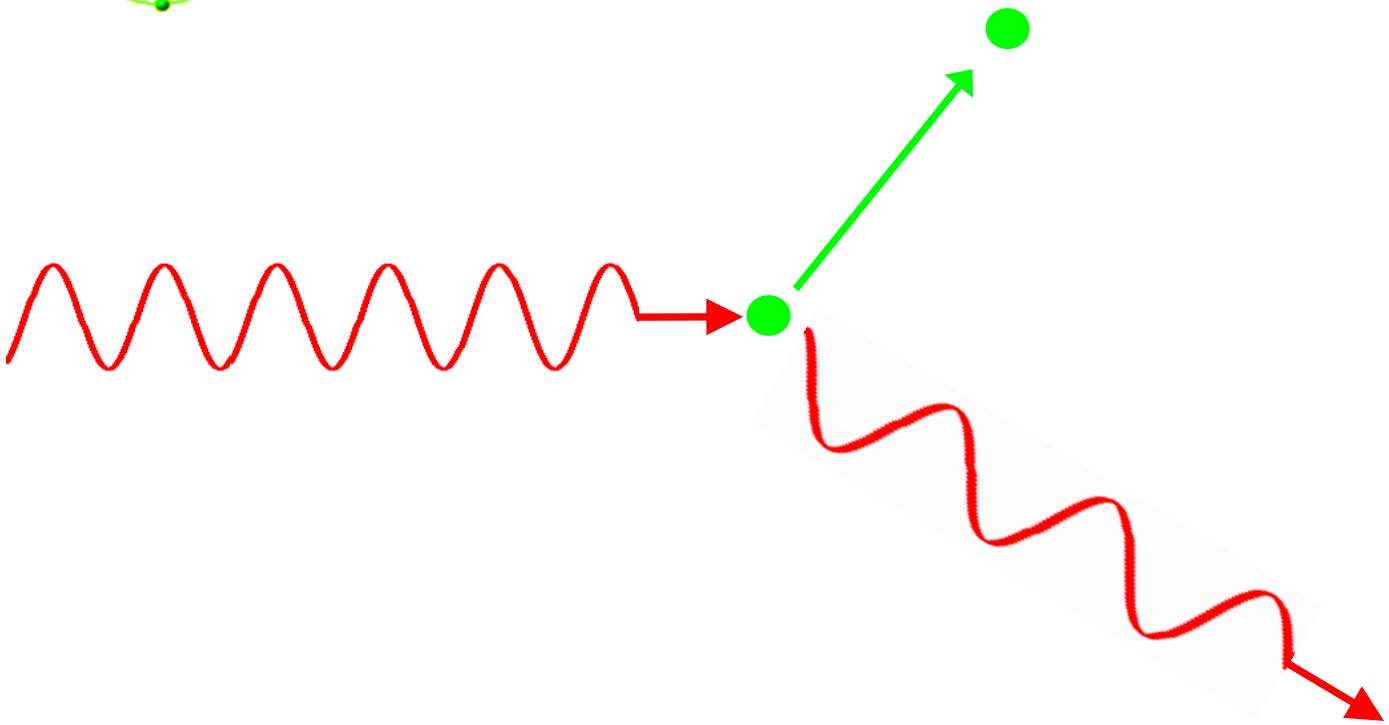


X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Compton (incoherent) scattering



?!



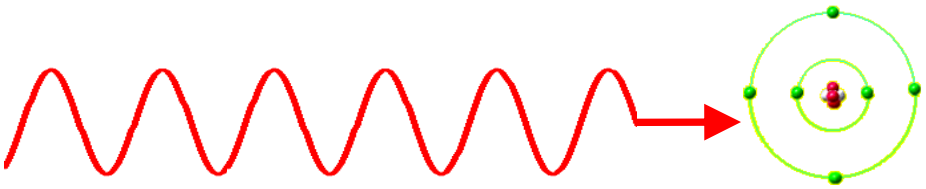
X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Incoherent – part of X-ray background in images.

Also a small proportion of total scattering: 8% at 1Å

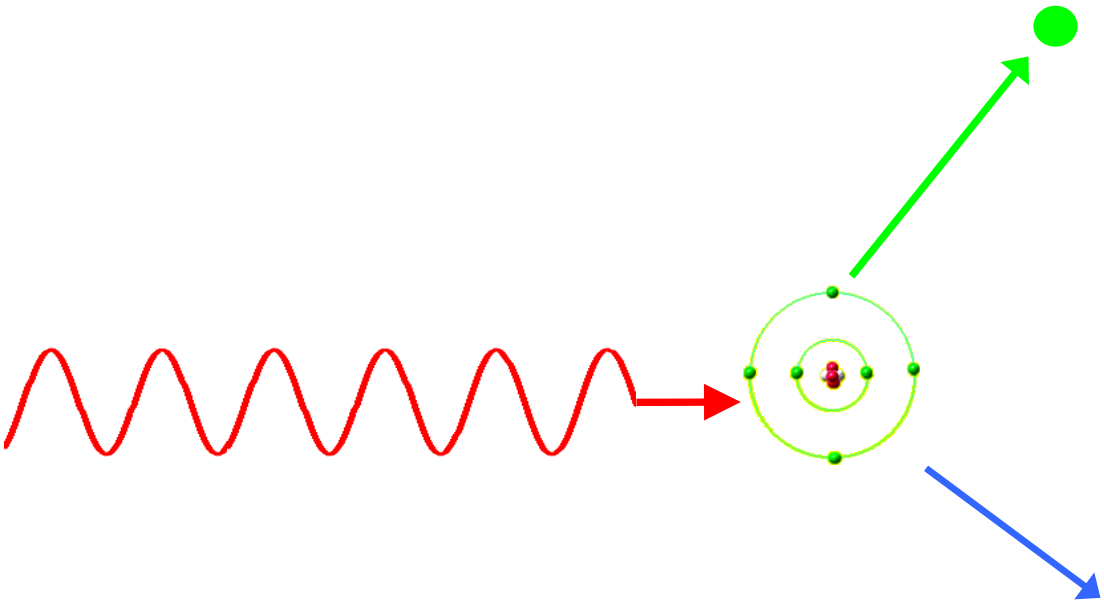
Photoelectric Absorption

84% at 1Å



INELASTIC.

Photoelectric Absorption



84% at 1Å

INELASTIC.

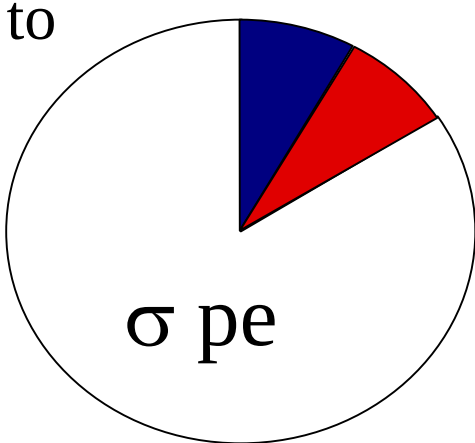
X-ray transfers all its energy to an atomic electron, which is then ejected.

Each 12 keV primary photoelectron can give rise to up to 500 ionisation events.

Atom can then emit a characteristic X-ray or an Auger electron to return to its ground state.

$$\sigma tot = \sigma\ pe + \sigma\ inc + \sigma\ coh$$

$$84\% + 8\% + 8\%$$



Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=$10^{-28}m^2$]

A few heavy atoms can make a big difference.

.

H

[Ravelli *et al.*, JSR,(2005) 12]

Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=$10^{-28}m^2$]

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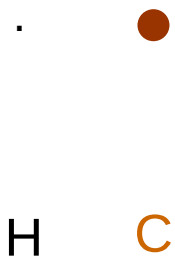

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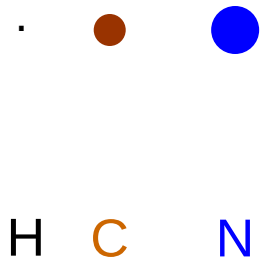

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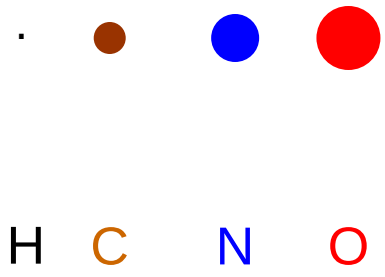

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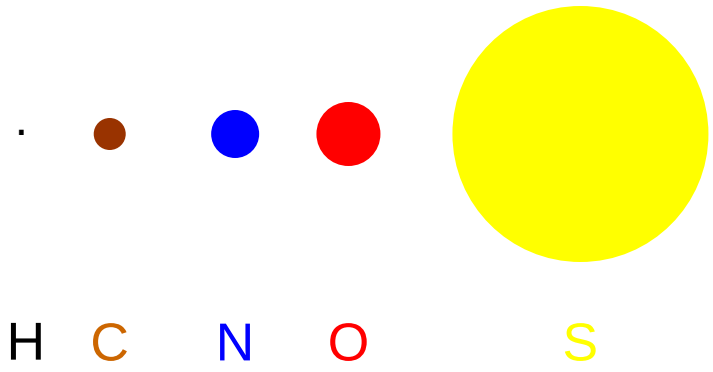

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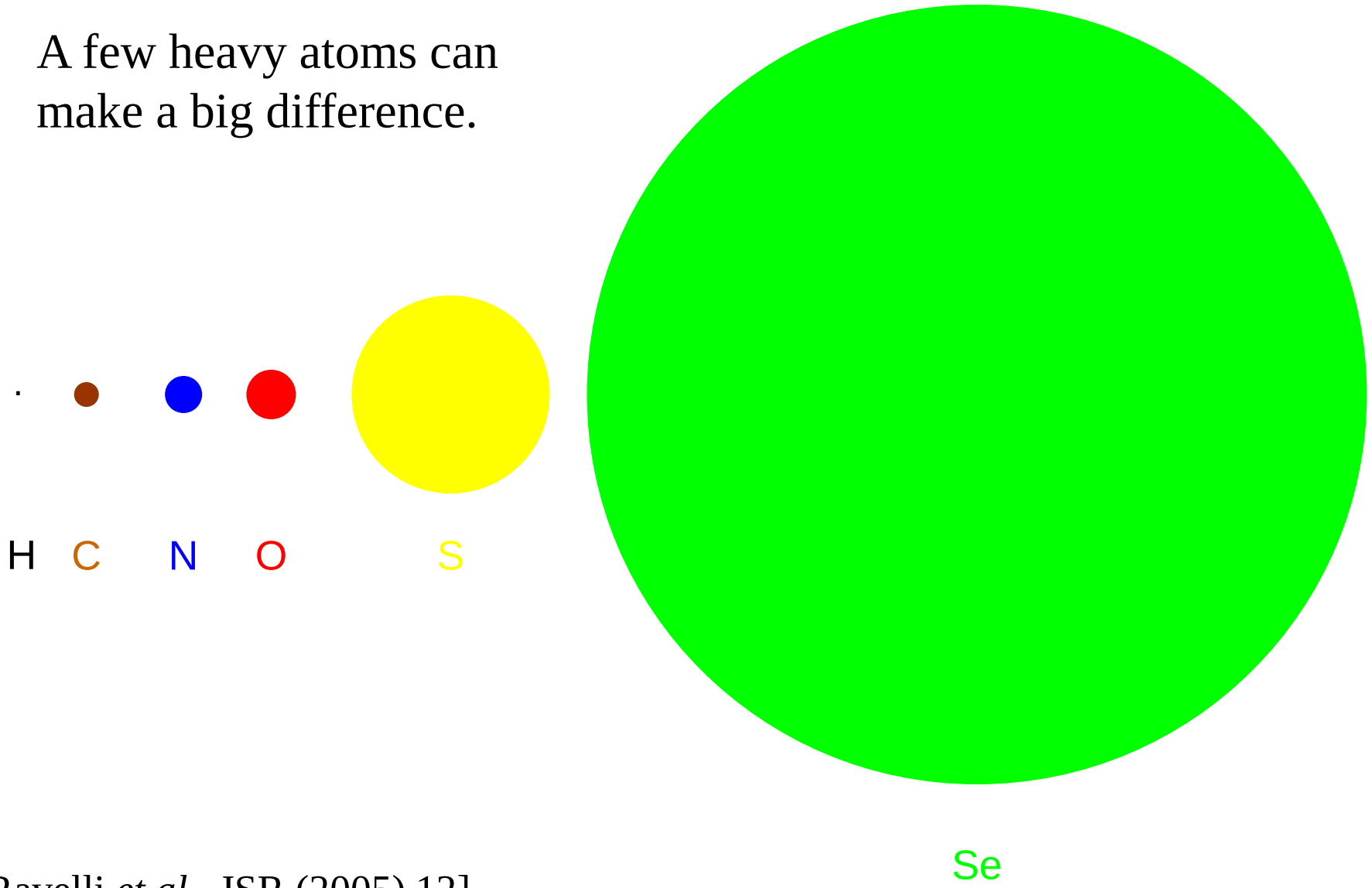
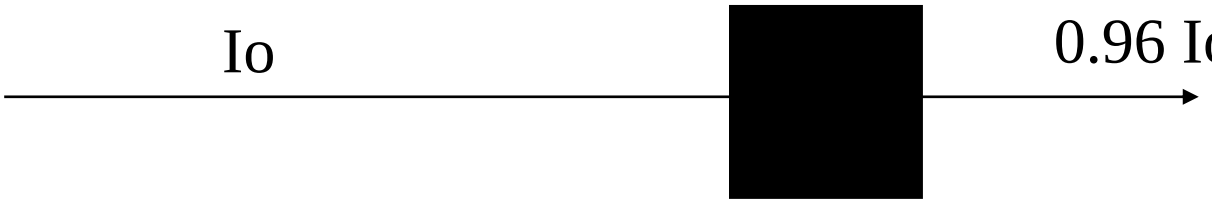


[Ravelli *et al.*, JSR,(2005) 12]

Beam absorption (λ=1Å) by a protein crystal

Native HEWL 100 µm thick

Platinum derivatised (1/molecule)
HEWL 100 µm thick



N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

A few heavy atoms in the solvent can make a BIG difference to the absorption cross section and this the dose rate for the SAME flux.

e.g. Cacodylate buffer (arsenic, mass 75 cf selenium = 79)

BACK SOAKING to REMOVE

Non-specifically bound heavy atoms

e.g. a brominated DNA-protein complex will radiation damage much faster than a native crystal, and will de-brominate during data collection [Ennfar et al, Acta Cryst D (2002) 1263-1268].

Radiation damage:

The Plan:

- What are the symptoms?
- What is it?
- **Why do we care? Effect on MAD/SAD.**
- How do we calculate the Dose?
- What do we know/would like to know?

Effect on MAD/SAD.

- Failure of structure determination

(Multi-wavelength anomalous dispersion MAD)

due to creeping non-isomorphism –

a) cell expansion and

b) movement of molecule in unit cell

c) structural changes DURING experiment.

Non-isomorphism: DISASTER!

Crick and Magdoff (1956) showed that for a 0.5% change in all three unit cell dimensions of 100Å, the intensity would change by

15% at 3Å

for general reflections

[Crick and Magdoff (1956) Acta Cryst **9**, 901-908]

i.e. **MAD/SAD phasing** signals (<5%) washed out completely.

Radiation damage:

The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- **How do we calculate the Dose?**
- What do we know/would like to know?

DOSE Postulate:

- There is a MAXIMUM dose (Joules/kg = Gy) which protein crystals can tolerate which depends only on the PHYSICS of the situation.

- Crystal might not reach that limit due to chemical factors, but it will not last BEYOND the limit.

- Need to be able to calculate the DOSE:

RADDOSE

V1: Murray, Garman & Ravelli (2004) JAPC, 37, 513-522
V2: Paithankar, Owen & Garman, (2009) JSR 16, 152-162
V3: Paithankar & Garman (2010), Acta D 66, 381-388

Typical MX experiment

A dose of 1 MGy will be absorbed in 10 s by a 100 μm cubed metal free crystal in a 100 μm x 100 μm

12.4 keV (1 Å) X-ray beam

flux 10^{12} photons s^{-1}

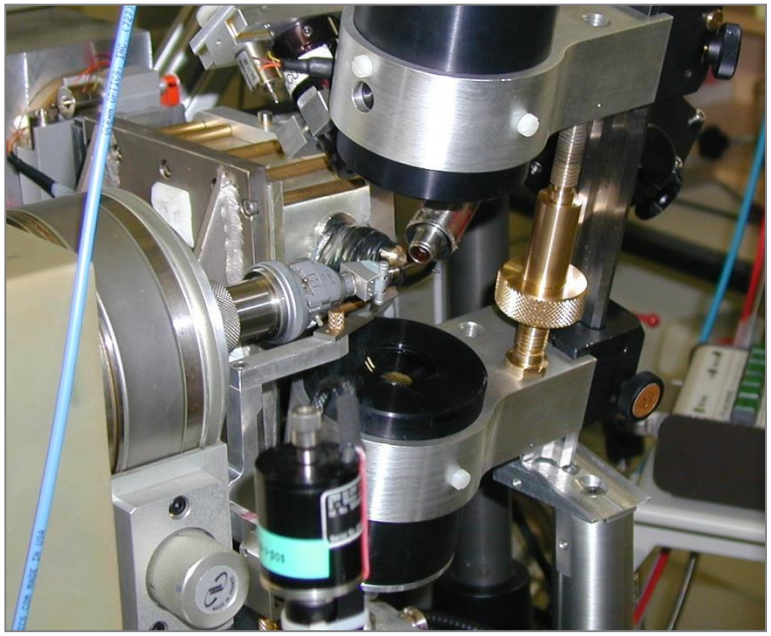
3 Gy

MX at 100 K: for above example,
The 30 MGy dose ‘limit’ is reached in approx 5 mins

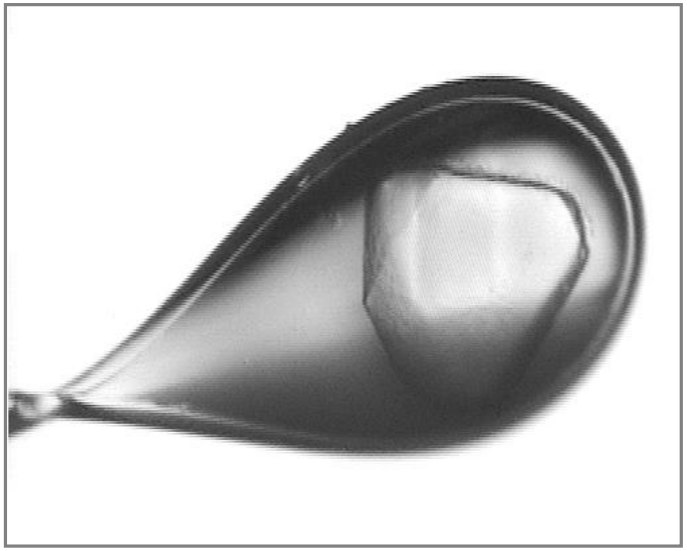
Dose calculation

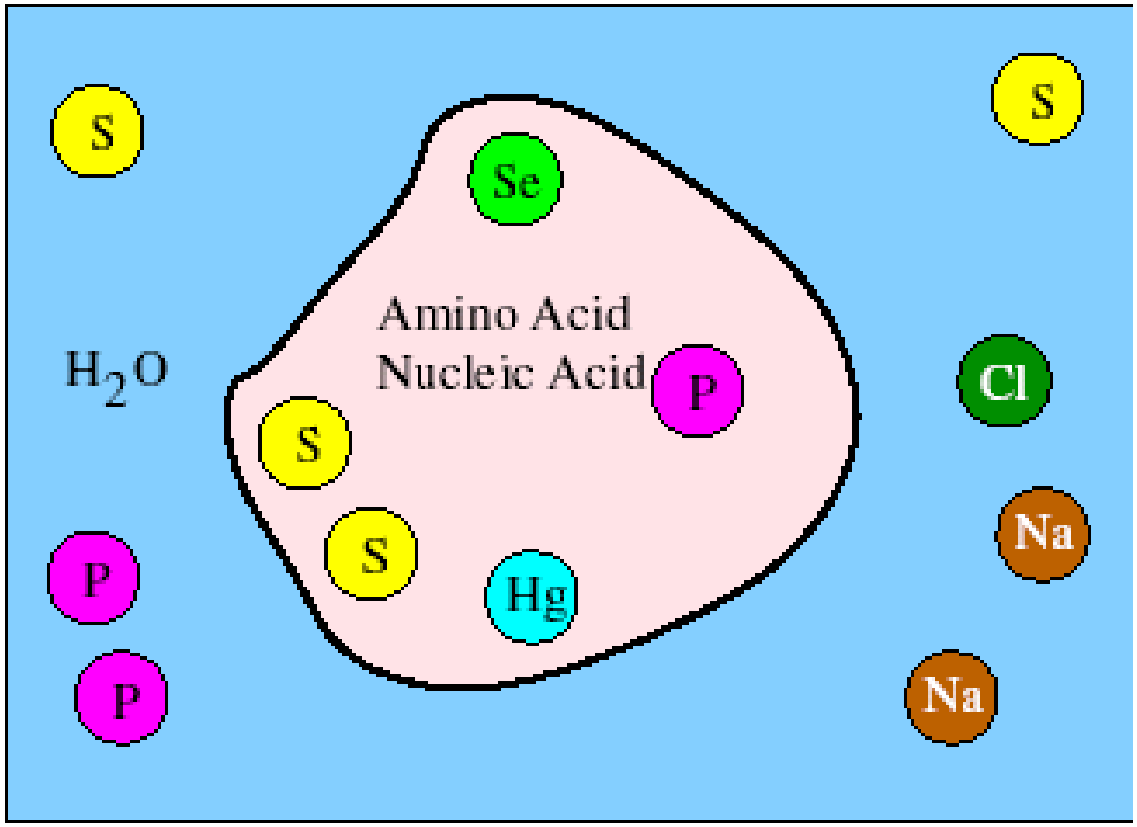
To find the energy deposited per unit mass in the crystal, need to characterise two things:

The Beam



The crystal





Number of Amino Acids

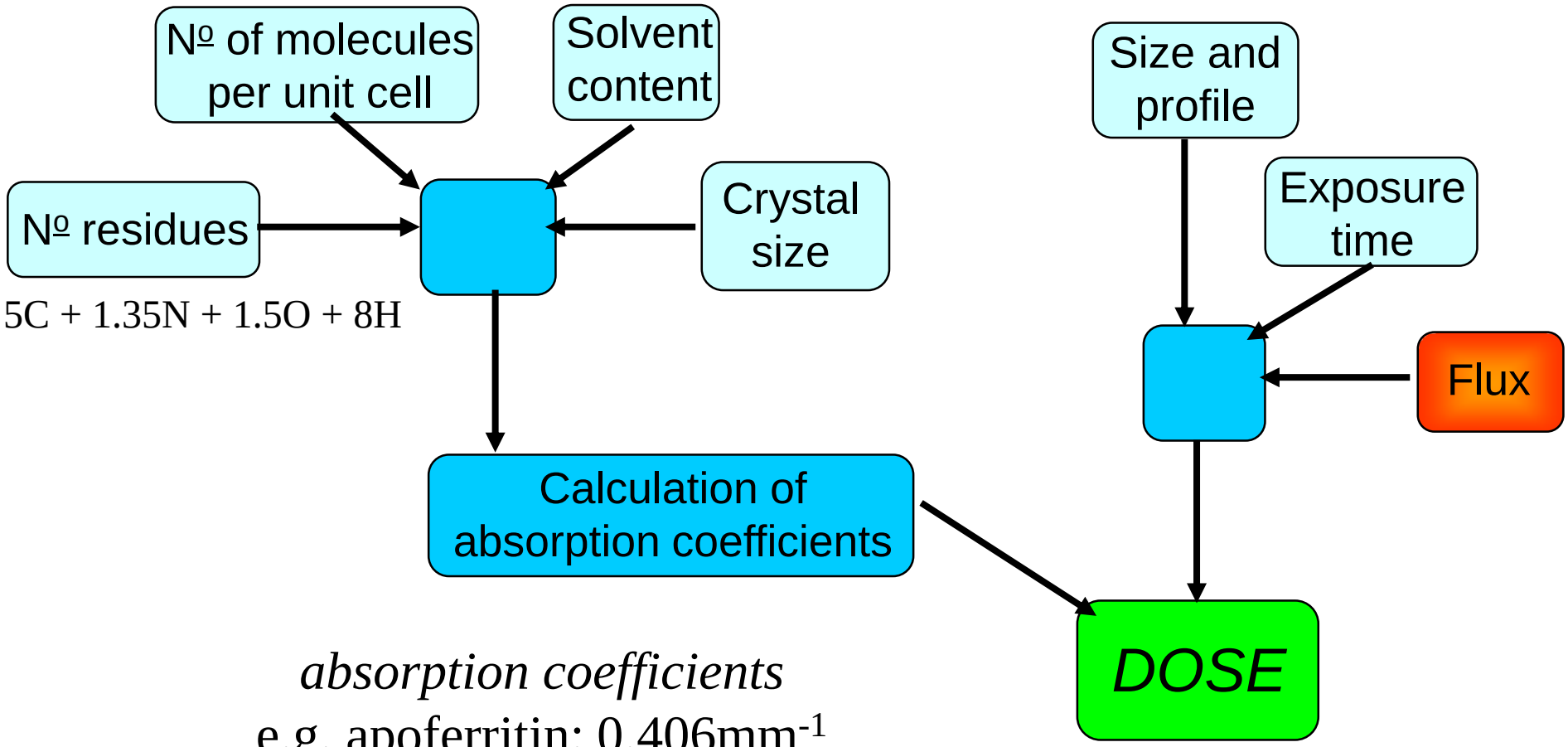
‘HA’ atoms per monomer, *e.g.* S, Se, Hg

Solvent - concentrations of components, *e.g.* Na^+, Cl^-

Calculating Dose
(*RADDOSE*)

Crystal Characteristics

Beam Characteristics



absorption coefficients

e.g. apoferritin: 0.406mm^{-1}

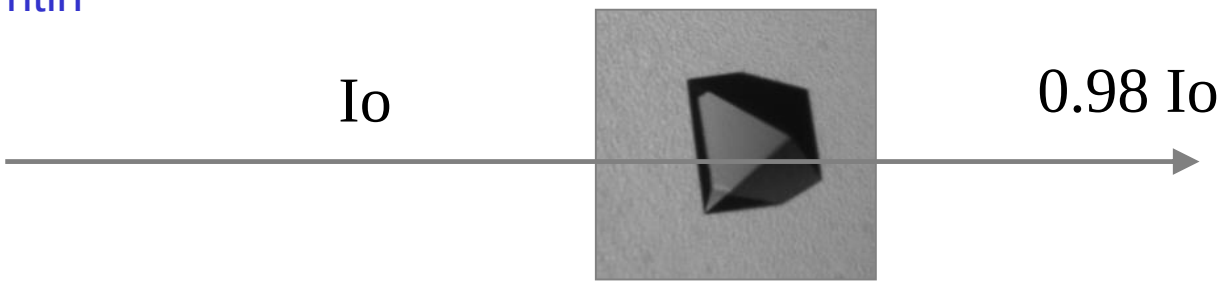
holoferritin: 1.133mm^{-1}

Ferritin

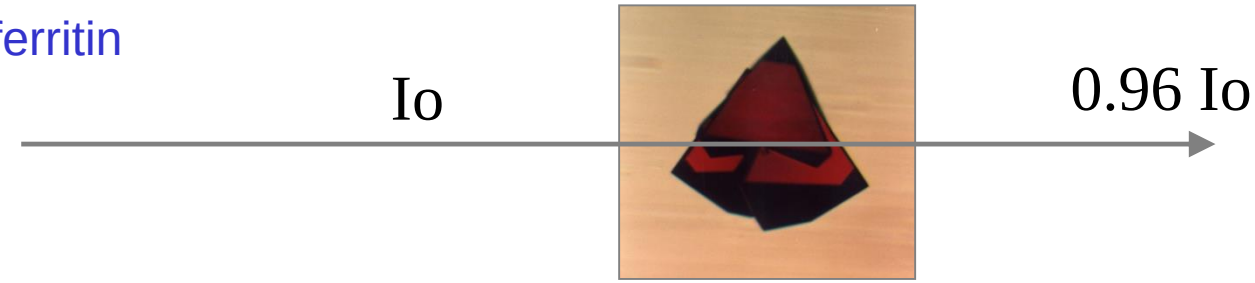
N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

The heavy atom ($z \geq 16$) content of a crystal is not crystallographically defined.

Apoferritin



Holoferritin

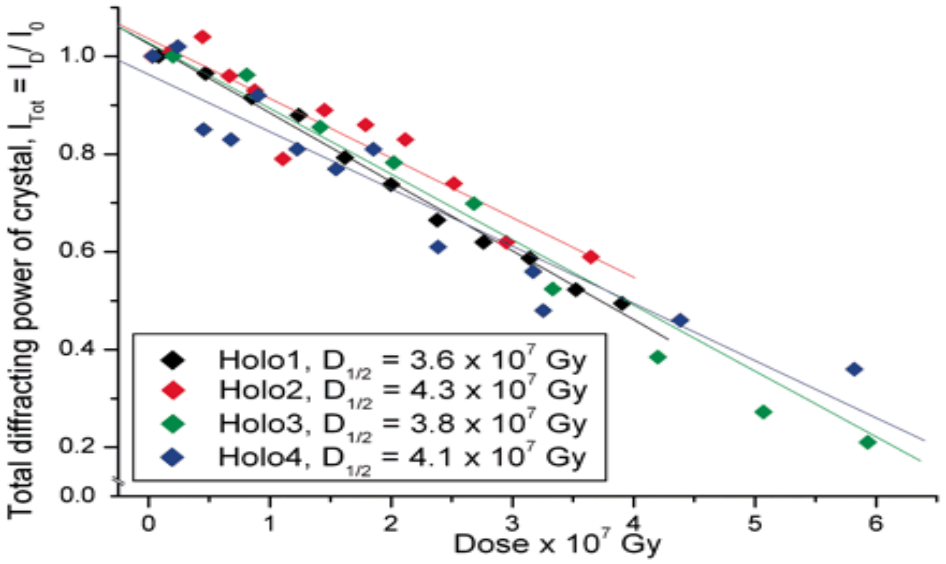


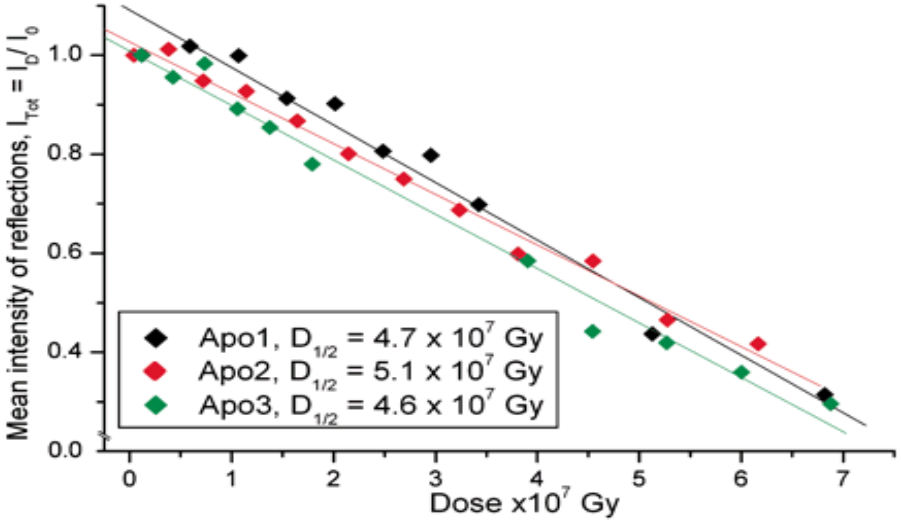
λ=1Å
100 μm thick xtal

Quantification at cryotemperature

- Holoferritin and Apoferritin as model
  - Absorption coefficient differs by factor of 2
- Linear dependence on dose
- $D_{1/2} = 4.3 \times 10^7$ Gy

Where $D_{1/2}$ is dose to half the intensity lost





Experimental Dose Limit

$$I_0 \times 1/2$$

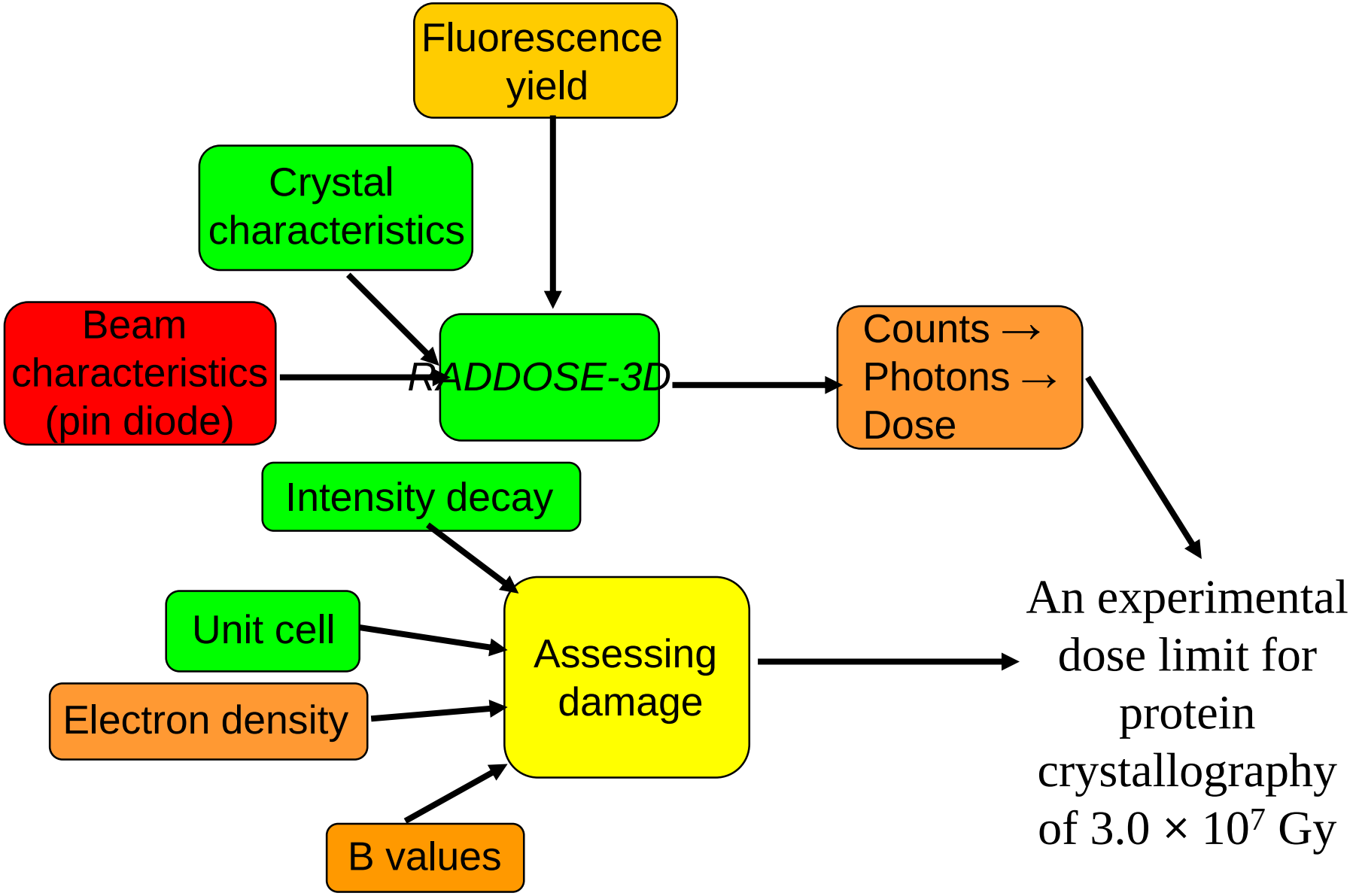
$$D_{1/2} = 4.3\ (\pm\ 0.4) \times 10^7\ \text{Gy} = 43\ \text{MGy}$$

Henderson limit:

$$D_{1/2} = 2.0 \times 10^7\ \text{Gy} = 20\ \text{MGy}$$

Howells et al (2005) recommended a resolution dependent limit of 10 Gy/ Å

Radiation damage to protein crystals: www.raddo.se



Experimental Dose Limit (100K)

For $I_0 \times 1/2$

$D_{1/2} = 4.3 (\pm 0.4) \times 10^7$ Gy = 43 MGy

BUT

Suggested limit to retain biological `fidelity'

$I_0 \times 0.7$

$D_{0.7} = 3.0 \times 10^7$ Gy =**30 MGy**

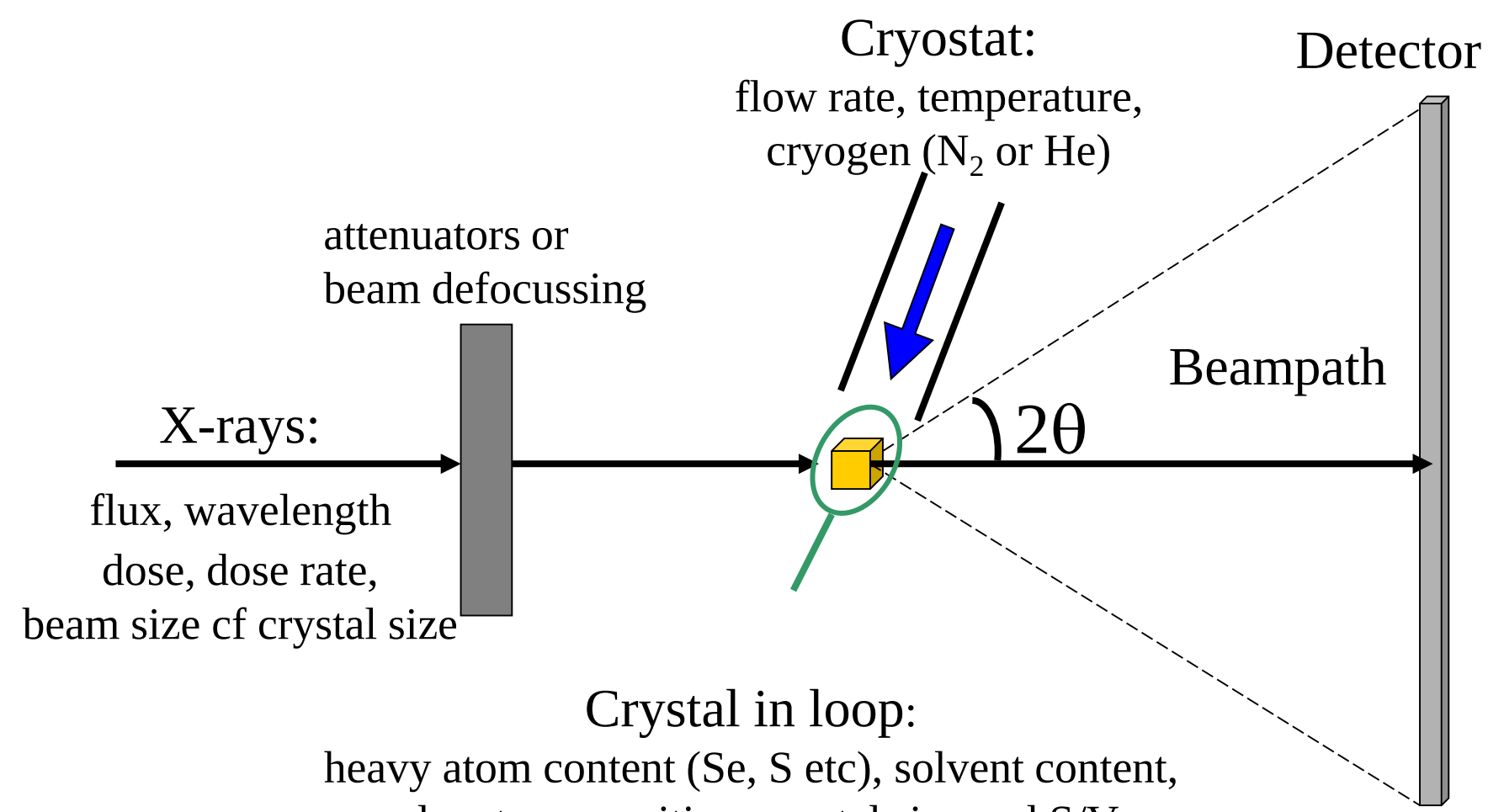
$D_{0.7}$ for ferritin corresponds 107 photons/unit cell

Robin Leslie Owen, Enrique Rudiño-Piñera, Elspeth F. Garman.
PNAS (2006) 103, 4912 - 4917.

Radiation damage:

The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- How do we calculate the Dose?
- **What do we know/would like to know?**



Crystal in loop:

heavy atom content (Se, S etc), solvent content,
solvent composition, crystal size and S/V,
amount of residual liquid around crystal,
choice and concentration of cryoprotectant agent,
time in cryobuffer, flash-cooling method (stream or liquid),
cryogen used to flash-cool, amount of crystal manipulation,
humidity, speed of experimenter when flash-cooling.

[Garman, Current Opinion of Structural Biology 2003, **9,** 545-551]

PROBLEM: how do we know that we are making any difference?

- In order to investigate the effects of various parameters on the radiation damage process, we need a robust radiation damage METRIC which is preferably ON-LINE during the diffraction experiment.

No unanimous metric currently/ results from different metrics do not agree.

- Structural changes occur before degradation of diffraction quality is obvious.

Work so far / ongoing:

- Lower the cryogen temperature? 40K? 50K? 16K? 140K (no!). Less than a factor of 2 improvement.
- Lower the wavelength? Lots of anecdote + now some systematic results: no effect on damage rate.
- Unit cell expansion as a metric? No!
- Change/ regulate the dose/dose rate regime? No, not at current!

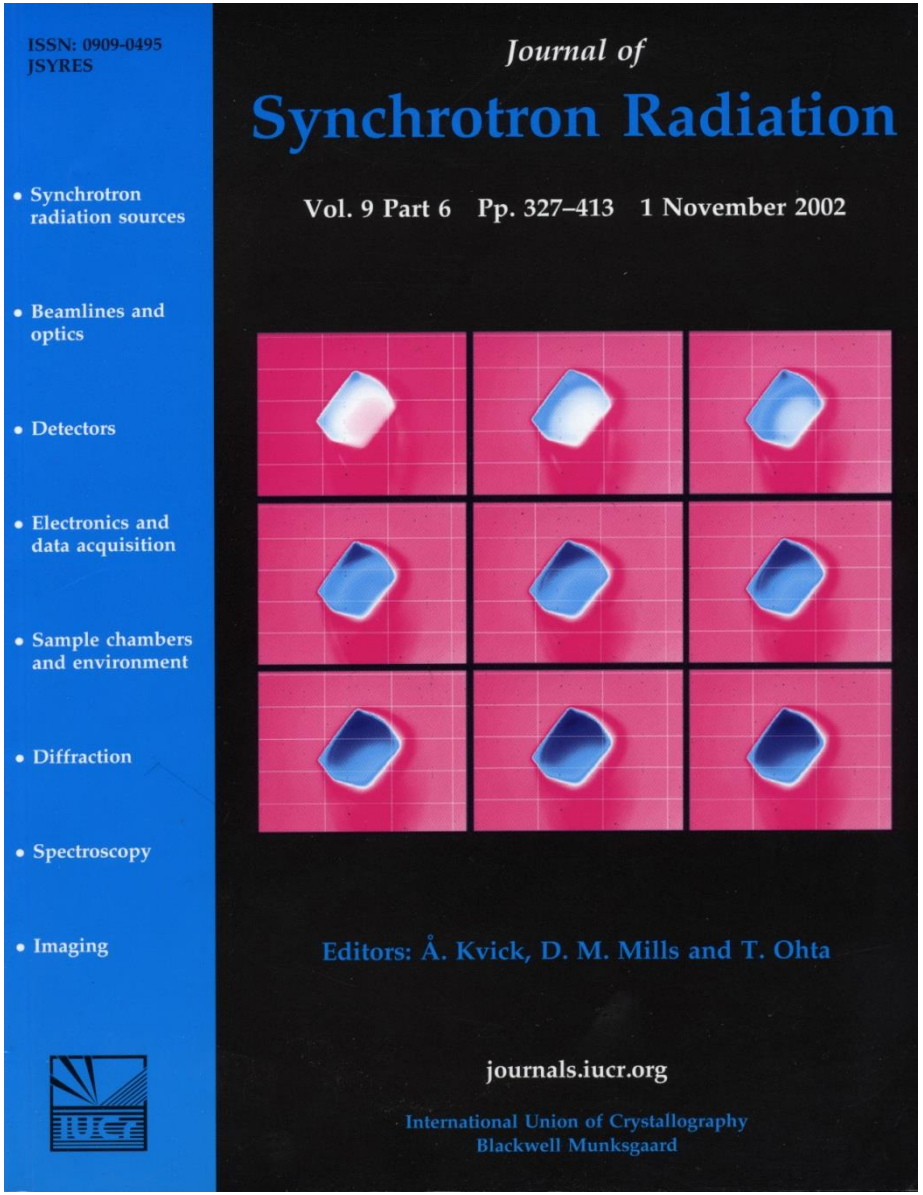
- Effect on MAD/SAD? Order of data collection?
- Minimum crystal size? Several papers. (see Holton 2009)
- Beam heating. Not a big factor at current flux

Work so far / ongoing:

- X-ray absorption – important parameters defined.
- Remove oxygen? Nothing yet.
- Radiation damage Induced Phasing (RIP)
- Software developments – big progress.
- Add radical scavengers: results disagree.
- Biological implications/applications to mechanistic studies. Now many.
- Room temperature studies: dose rate effects? Results disagree.

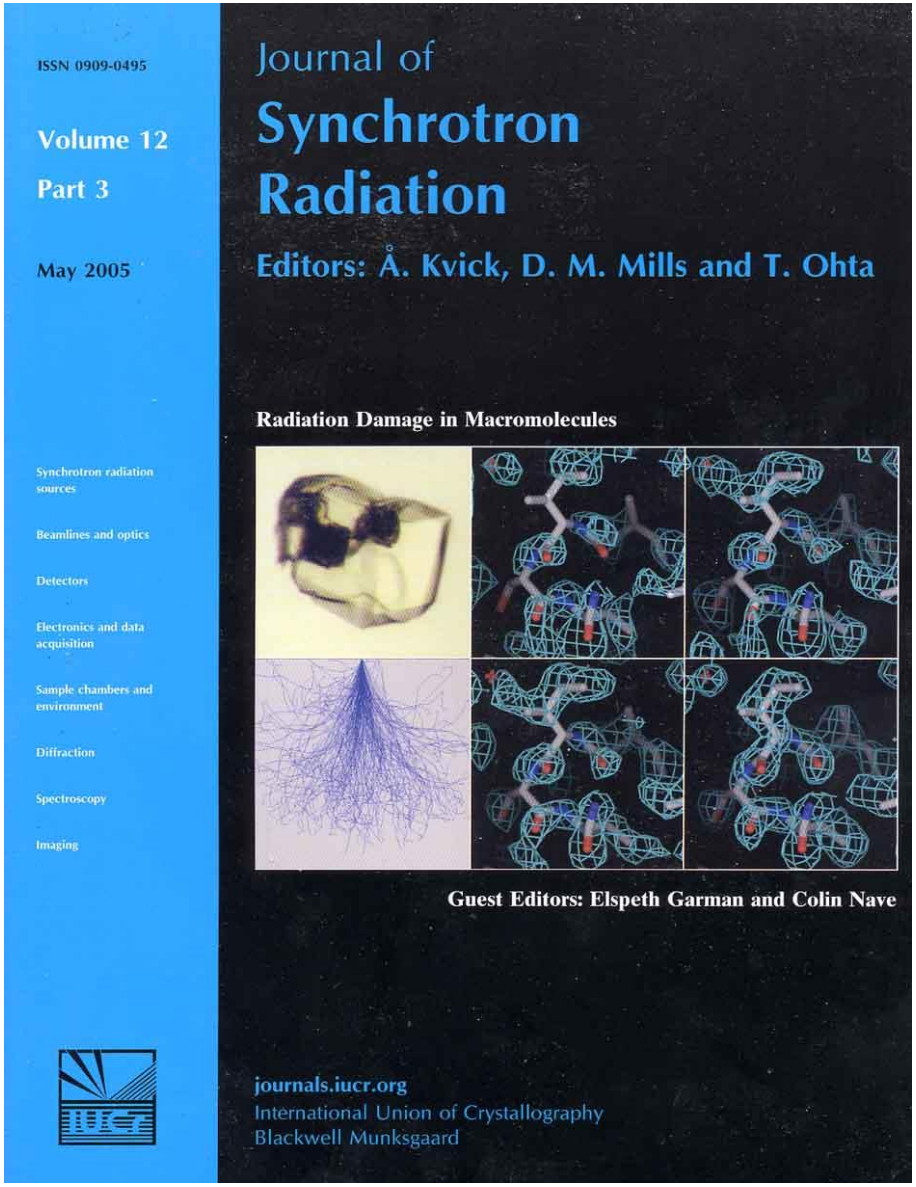
- N.B. Need for **systematic statistically significant** experiments.
- Series of Radiation Damage Workshops

RD2: Dec 2001



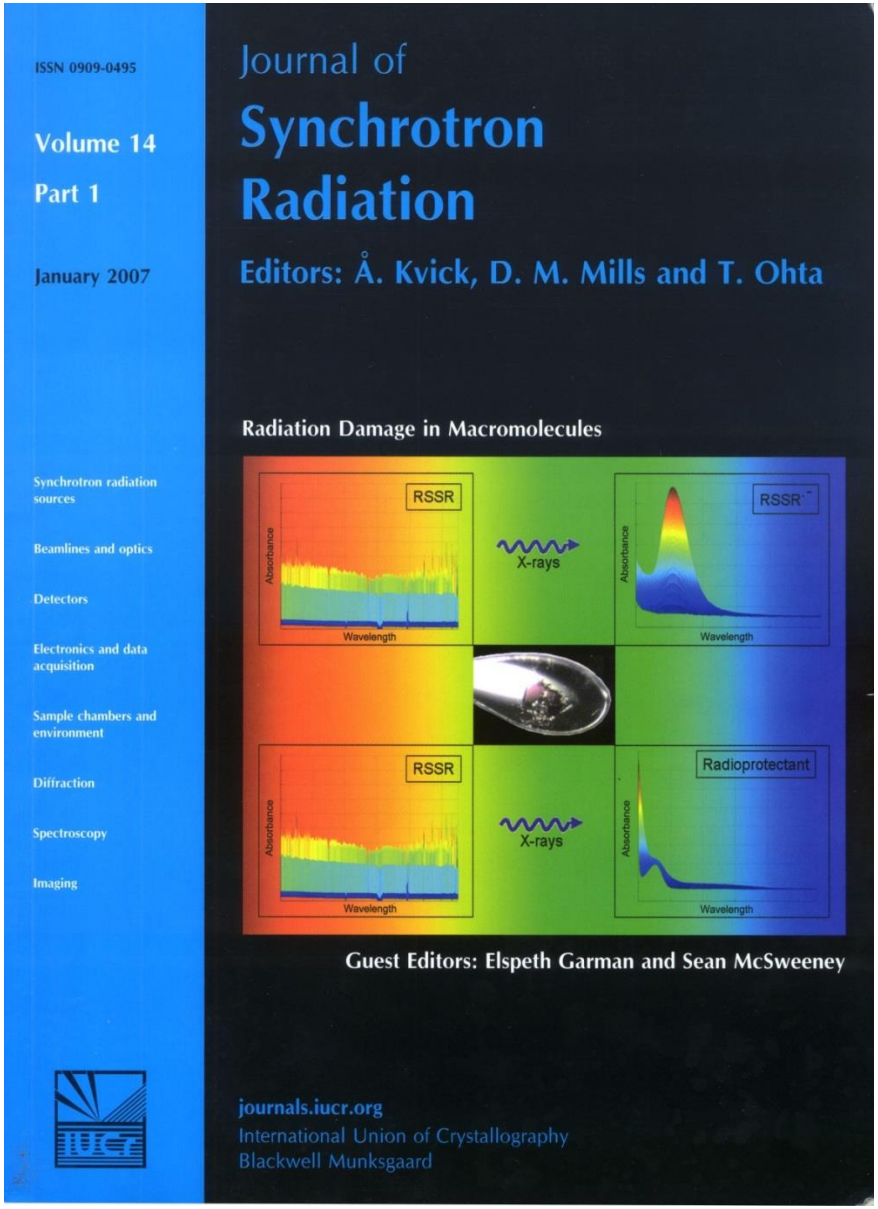
JSR, Nov 2002, 8 papers

RD3: Nov 2003



JSR, May 2005, 9 papers

RD4: March 2006

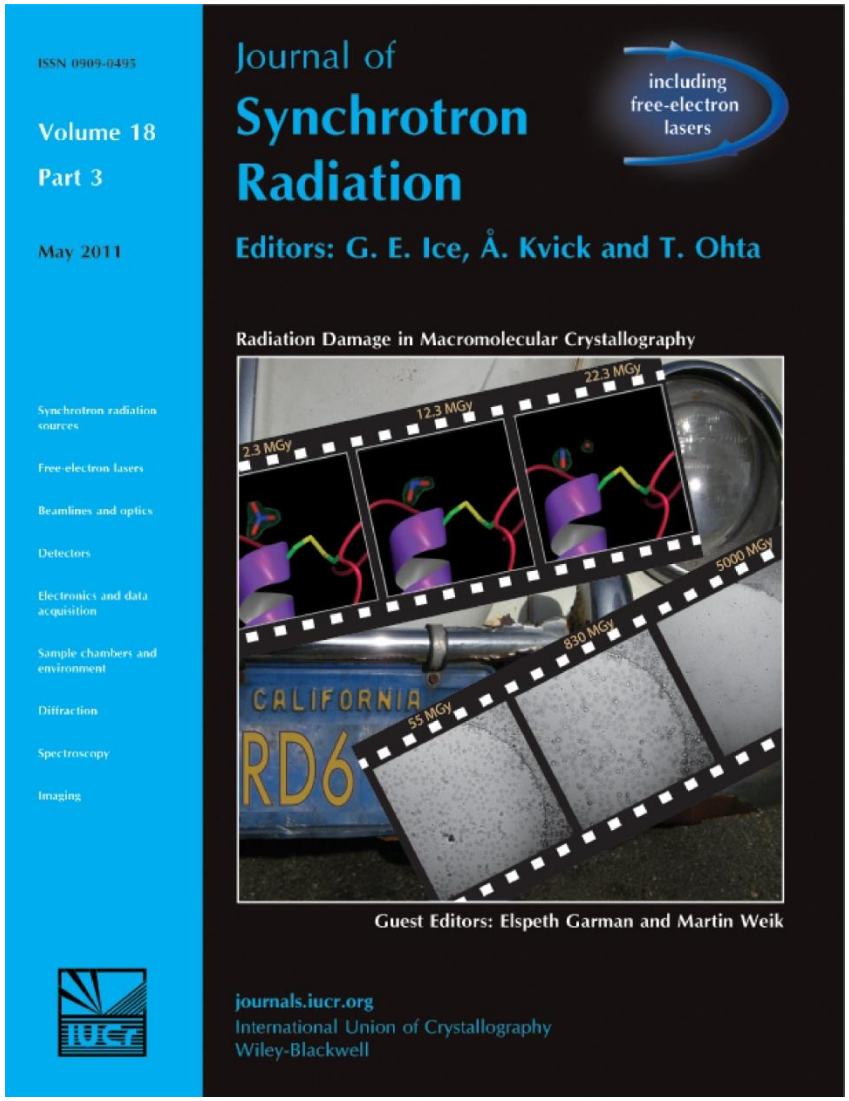


JSR, Jan 2007, 14 papers

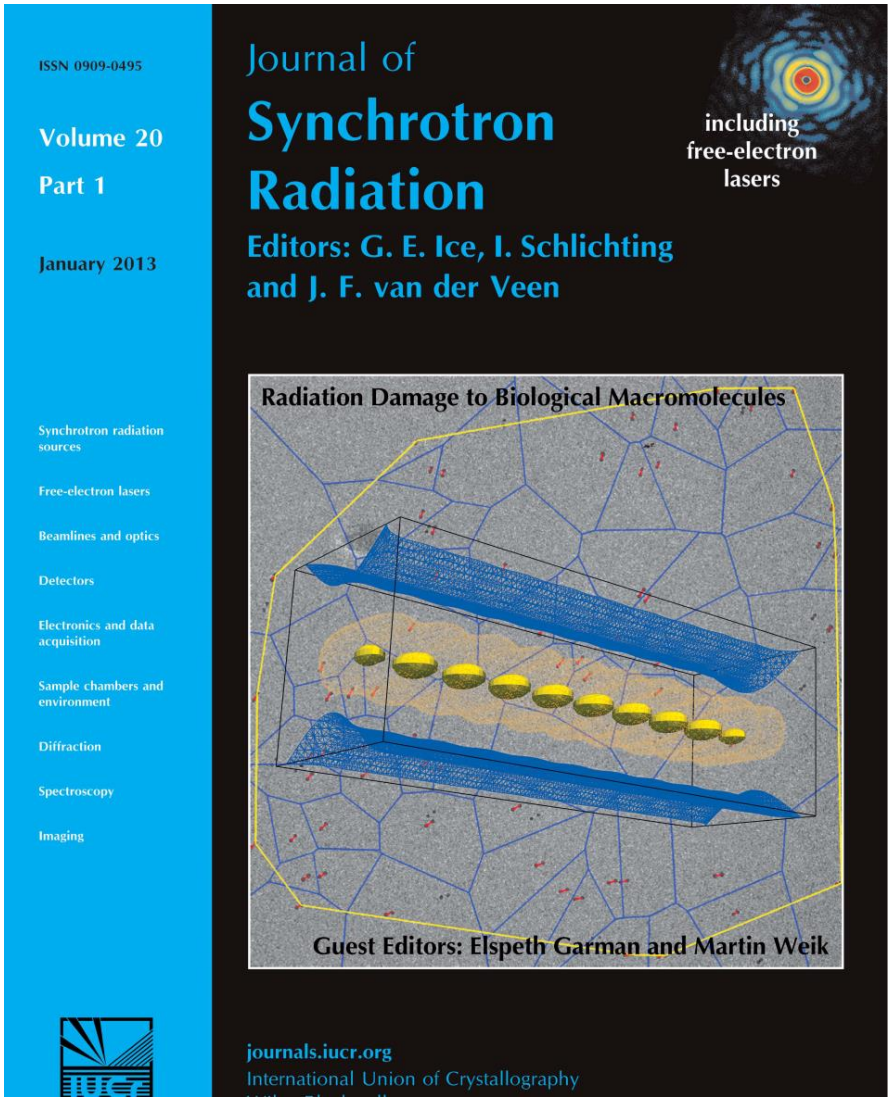
RD5 March 2008

JSR, March 2009, 8 papers

RD6: March 2010



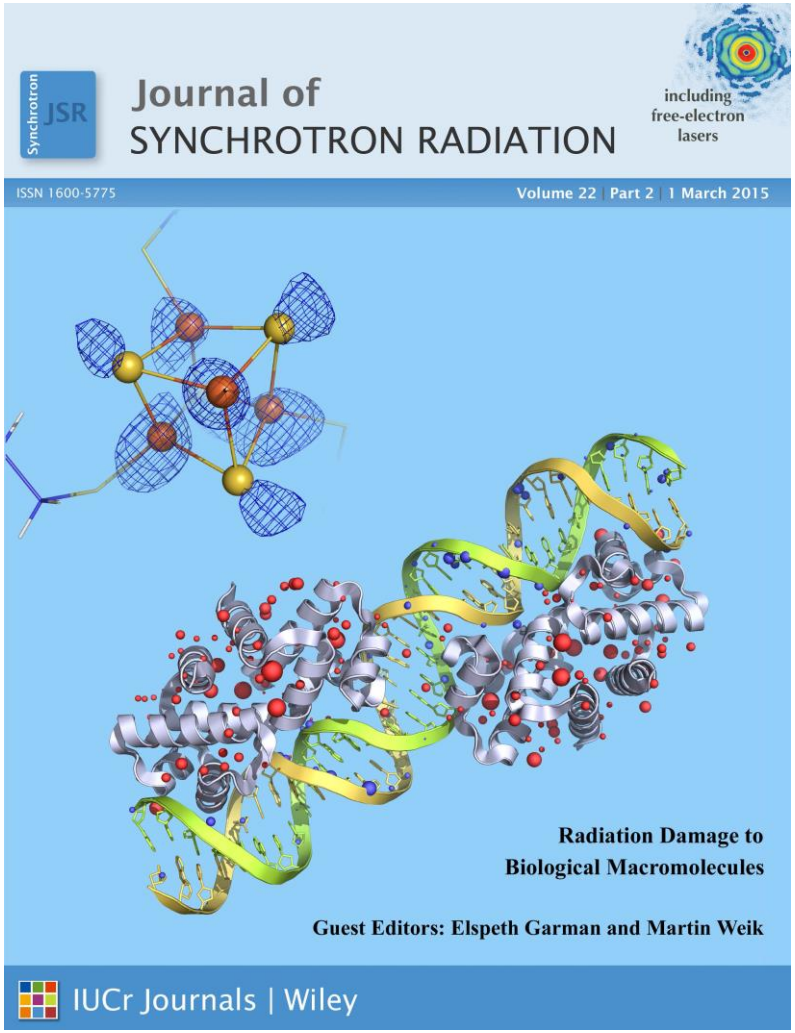
RD7, March 2012



JSR, May 2011, 10 papers

JSR, Jan 2013, 6 papers

RD8: April 2014



JSR, March 2015, 8 papers

RD9: 9-11 March 2016
MAX IV Lund

More JSR papers
in 2017 from RD9??

General summary in:
Garman Acta D (2010)
66, 339-355

Also a Beginner's guide to RD in Holton (2009) JSR 16,133-142

Summary 1: what can YOU, the experimenter do?

- Do not be afraid to merge data taken from different isomorphous crystals which all had lower doses.
- Back soak non-specifically bound heavier atoms out of your crystals.
- Be ‘absorption aware’ of the contents of your crystal (e.g. Se and buffer) and if possible, avoid cacodylate buffer (arsenic mass=75).
- Match beam size to crystal size

Summary 2: what can YOU, the experimenter do?

- Scavengers: try electron scavengers at 100 K (nitrate/ascorbate/benzoquinone).

- Dose ‘spreading’: use a tophat profile beam if possible. Consider Helical/Translational data collection.

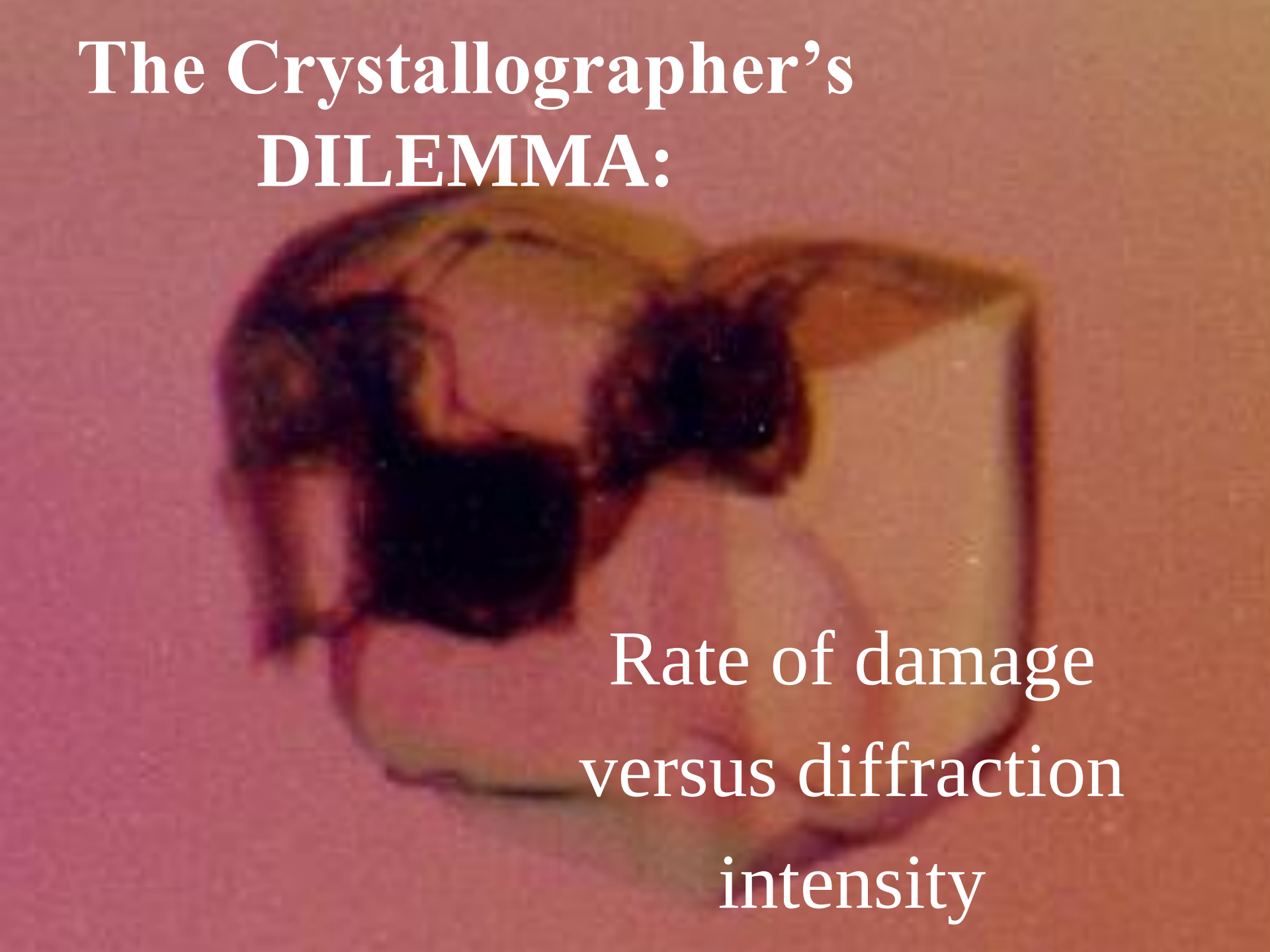
- So you can estimate the dose, ASK at the beamline:
  - What is the flux today at this energy and with this slit size (‘flux density’)?
  - What is the beam profile today at this beam energy? FWHM in *x* and *y*?

Current status: radiation damage in protein crystals

- Understand a lot more than twelve years ago, but still not nearly enough…
- Understand how to do experiments better.
- Research has prompted some very exciting new approaches.
- Many complementary methods now being used on the problem in concert with crystallography
- Experiments must involve more than one sample (!) to get statistically significant results: labour intensive and time consuming. Also MUST know incident FLUX density…
- **Radiation damage has DEFINITELY become a mainstream concern**

Still the first day and my brain is already over full…

The Crystallographer's DILEMMA:



Rate of damage versus diffraction intensity